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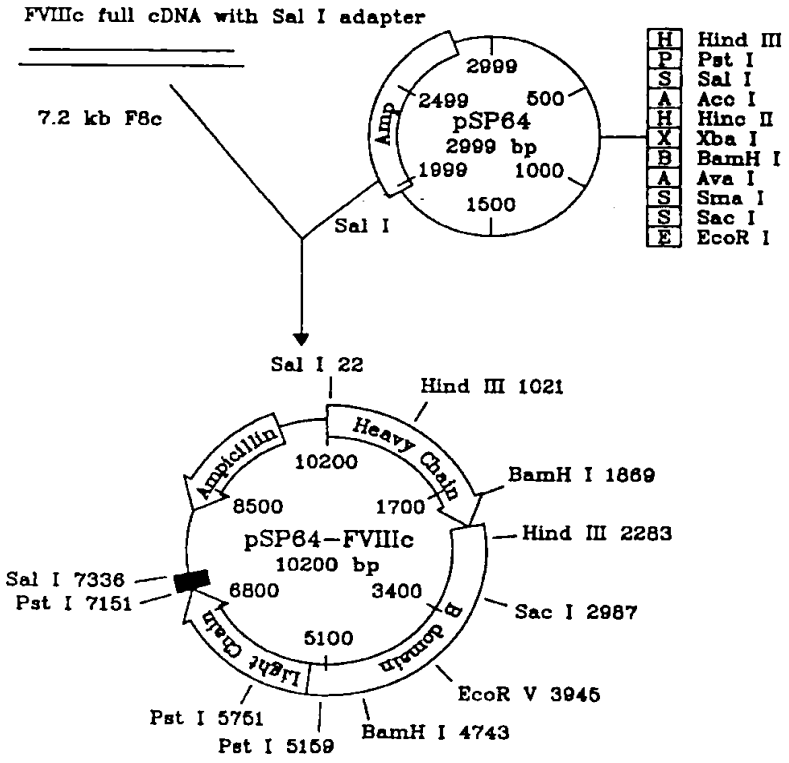
(54) Title: TRANSGENIC PLANT-DERIVED HUMAN BLOOD COAGULATION FACTORS

(57) Abstract

A composition is provided for transgenic plants and transgenic plant derived human coagulation factors *capable of eliciting an activation response in human blood clotting pathways and therefore* useful for the treatment of human beings diagnosed to be deficient in blood clotting factor proteins. Such proteins may be manufactured by methods resulting in viral free production using both whole plants and plant cell cultures. Also provided are expression vectors for the proper transformation of plant tissue for the production of such factors, as well as transformed plant cells and processes for producing human coagulation factors using plant molecular biology techniques.



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TRANSGENIC PLANT-DERIVED HUMAN BLOOD COAGULATION FACTORS

FIELD OF THE INVENTION

The present invention relates generally to transgenic plant-derived human coagulation factors. More specifically, the invention relates to producing human-like blood coagulation factors (e.g, factors VIII, IX, XIII, thrombin) from transgenic plant cells encoded to produce human coagulation factors. As used herein, the terms "human factor" and "human coagulation factor" are interchangeable and refer to human-like proteins possessing human factor-like procoagulant or coagulant activity derived from transgenic plants.

BACKGROUND OF THE INVENTION

Given the technological advances in recombinant DNA technology made over the past decade it has become common practice to introduce new genetic material into plant cells, plants, or plant tissue to establish new traits that enhance the value of the plant or plant tissue. The present invention relates to the introduction of genes or DNA encoding human blood coagulation factors into plants. The present invention also relates to the production of active human-like blood clotting proteins from transgenic plant materials which provides a cost effective means for producing viral free, human-like blood coagulation factors. The invention further relates to the use of plant-derived human factors for blood factor replacement, wound healing or other therapeutic applications contemplated for human blood coagulation factors. When used hereinafter, the term "plant" shall refer to the plant itself, cells or tissues derived from the plant, seeds, cuttings, or other plant-derived structure. Both monocotyledonous and dicotyledonous angiosperm plants are included within the definition of "plant".

General Overview of Blood Coagulation Process and Problems

Damage to the human vascular system leads to the participation of many physiological processes that are important in controlling blood loss. First, the platelet adhesion process occurs, where platelets become sticky and bound to the endothelial connective tissue structures, leading to platelet plug formation. Second, the platelet release reaction gives rise to vasoactive amines, such as serotonin which causes vasorestriction of the injured vessel. The third effect is the triggering of the coagulation process, involving a cascade of proteins in both the intrinsic and extrinsic systems, to arrest bleeding. The fourth important effect is the activation of the fibrinolytic system, which leads to the degradation of the fibrin clot, healing and regeneration of the vessel wall. This particular invention is focused on production of proteins involved in the coagulation cascade. For a general review of blood coagulation, see "Basic Mechanisms in Blood Coagulation," by Davie et al. (1975. Ann Rev Biochem 44:799) and "Blood Coagulation," by Bithell in *The Normal Hematopoietic System* (Bloom and Thomas, Eds.. Churchill. Livingston, N.Y., 1981, pp. 566-615).

The primary role of the coagulation cascade is to stabilize the initial platelet plug. This system consists of over a dozen interacting proteins present in plasma as well as released or activated cellular proteins. Each step of the cascade involves the activation of a specific inactive (zymogen) form of a protease to the catalytically active form. The zymogen form of each protein, with a few exceptions, is assigned a Roman numeral designation, while the activated form is designated by a Roman numeral followed by a subscript "a". The activated form of the protease in each step of the cascade catalyzes activation of the subsequent protease in the cascade. In this fashion, a small initial stimulus, either via contact or proteolysis, is catalytically amplified at each step, culminating in a burst of thrombin which catalyzes the formation of insoluble fibrin needed for clot (platelet plug) stabilization.

Blood coagulation proteins participate in two closely related clotting mechanisms that lead to the formation of the fibrin clot. These mechanisms are referred to as the *intrinsic* and *extrinsic* coagulation pathways (**FIG. 1**). The intrinsic coagulation pathway is initiated by contact between factor XII and an active surface (e.g., unbroken skin,

articular cartilage, vascular basement membrane, sebum, long-chain fatty acids, etc.) or through fluid-phase proteolysis via kallikrein or some other enzymatic activator. Subsequent activation of factor XII initiates a series of reactions involving factors XI, IX, VIII, prekallikrein, high molecular weight kininogen (HWMK), and platelet factor 3 (PF-3), which lead to the activation of factor X. The extrinsic coagulation pathway is initiated by interactions between tissue factor and factor VII in the presence of Ca^{2+}, leading to production of an enzyme that also activates factor X. This pathway does not require contact activation. However, proteolytic activity that evolves early in the contact phase of coagulation greatly enhances the activity of factor VII.

Subsequent steps in the blood coagulation process are referred to as the *common* pathway of coagulation because they are common to both the intrinsic and extrinsic pathways. This pathway involves factors X and V, PF-3, prothrombin, and fibrinogen and proceeds in essentially the same manner regardless of whether factor X is activated by factor IXa, PF-3, and factor VIIIa (intrinsic pathway) or by factor VIIa and tissue factor (extrinsic pathway). In the final step of the common pathway, soluble polymeric fibrin is stabilized into a non-soluble clot through interactions with factor XIIIa.

Specific coagulation factors of current or potential pharmaceutical value include factors VIII, IX, VII, XIII as well as thrombin, fibrin, and fibrinogen. Most notably, factors VIII and IX are respectively used in the treatment of the two most common hemophilic disorders, hemophilia A (classical hemophilia) and hemophilia B (Christmas disease). Hemophilia A is a sex-linked bleeding disorder, characterized by a deficiency in human coagulation factor VIII. Likewise, hemophilia B is a sex-linked bleeding disorder resulting from a deficiency of human coagulation factor IX. Approximately 80% of all hemophilia disorders are due to a deficiency of factor VIII. Both types of hemophilia clinically result in the lack of sufficient fibrin formation required for clot stabilization. Subsequently, hemophiliacs suffer chronic bleeding episodes resulting at sites of relatively weak clot formation. The relatively high frequency of factor VIII and factor IX deficiency when compared to other proteins in the coagulation cascade is due to their genetic linkage to the X-chromosome. A single defective allele results in hemophilia in males, who only have one copy of the X chromosome. Since deficiency of other coagulation factors is autosomally linked (i.e., generally, two copies are needed to

result in deficiency), hemophilia A and B are by far the most common hereditary blood clotting disorders, appearing almost exclusively in males.

Factor VIII is a highly specialized type of protein used primarily in the treatment of Hemophilia A as well as for other research and commercial applications. Factor VIII is a large glycoprotein that, when activated, functions in the blood coagulation cascade as a cofactor, along with calcium ions and phospholipid, in the factor IX mediated activation of factor X in the intrinsic coagulation pathway. It can be activated proteolytically by several coagulation enzymes, including thrombin. Plasma-derived factor VIII is in chronically short supply, therefore making this type of therapy highly cost prohibitive. In addition, resulting pharmaceutical products derived from plasma are highly impure, with a specific activity of 0.5 to 2 factor VIII units per milligram protein (one unit of factor VIII activity is by definition the activity present in one milliliter of normal plasma). Resulting plasma-derived factor VIII purity is typically lower than 1% by weight (Wood et al. 1984. Nature 312:330). The high level of impurities result in a variety of serious complications including transmission of hepatitis A, B, and C, human parvovirus and human immunodeficiency virus (HIV) pathogens. To circumvent difficulties with viral pathogen transmission associated with human plasma-derived factor VIII and to lower product cost, factor VIII has been successfully expressed in a variety of mammalian cell culture systems. Initially, recombinant factor VIII was produced in baby hamster kidney (BHK) cell lines, using the calcium-phosphate coprecipitation method (Simonsen et al. 1983. Proc Natl Acad Sci USA 80:2495, Wigler et al. 1979. Proc Natl Acad Sci USA 76:1373) for integration of the 7kb protein encoding region (Wood et al. supra 1984, Capon et al. 1997. U.S. Patent No. 5618788). Active recombinant human factor VIII has also been produced in Chinese hamster ovary (CHO) cells (Kaufman et al. 1988. J Biol Chem 263:6352) and monkey COS-7 cells (Toole et al. 1984. Nature 312:342, Truett et al. 1985. DNA 4:333). However, production of factor VIII using these mammalian cell lines does not eliminate the potential for transmission of human pathogens. Consequently, these cell lines require additional quality assurance testing during biosafety trials to safeguard against virus transmission.

Factor IX is the zymogen of a serine protease responsible for the activation of factor X in the intrinsic pathway of the coagulation cascade and is used primarily in the

treatment of Hemophilia B. Factor IX is activated directly by factor XI_a. Factor IX is normally synthesized in the liver and undergoes extensive post-translational modifications including glycosylation, γ-carboxylation of specific glutamic acid residues (DiScipio et al. 1979. Biochem. 18:899), and β-hydroxylation of a single aspartic acid residue (McMullen et al. 1983 Biochem Biophys Res Commun 115:8). Like factor VIII, commercially available factor IX is produced via both plasma purification and genetically modified mammalian cell culture.

Factor VII is a zymogen of an enzyme that forms a complex with tissue factor for the activation of factor X in the extrinsic coagulation pathway. Factor VII may be activated predominantly by kallikrein and factor XIIf under physiological conditions, although factor X_a, plasmin, and factor IX_a have been reported to also accomplish activation. Like factor IX, factor VII is synthesized in the liver and undergoes glycosylation as well as γ-carboxylation of specific glutamic acid residues (O'Hara et al. 1987 Proc Natl Acad Sci USA 84:5158). Therapeutically, factor VII_a was used initially for treatment of severe bleeding episodes in hemophiliacs who could not receive factor VIII due to immune system response. However, it is currently contemplated for use as a replacement therapeutic for factor VIII and factor IX in the treatment of hemophilia A and B, respectively. Factor VII may be more efficient in coagulation than these other factors since it travels directly to the site of the injury, rather than dispersing throughout the bloodstream. Recombinant factor VII is currently produced in its activated form (factor VII_a) in baby hamster kidney (BHK) cells.

Other blood coagulation factors, including thrombin and factor XIII, may be used directly in wound closure applications. Thrombin is a multifunctional protein catalyzing several key reactions in the coagulation cascade (Mann et al. 1988 Ann Rev Biochem, 57:915). In one reaction, this enzyme acts as a catalyst in the conversion of fibrinogen to fibrin, which is the main component in stable clots. In addition to this reaction, thrombin also activates platelets as well as factors V, VIII, IX and XIII. Therapeutically, thrombin is used primarily in tissue sealants, usually in conjunction with fibrinogen. Prothrombin, the zymogen for thrombin, requires γ –carboxylation of specific glutamic acid residues for activity. Prothrombin (Mr 72,000) is a vitamin K dependent glycoprotein protein that participates in the final phase of blood coagulation (Mann et al 1980 in: *CRC Handbook*

Series in Clinical Laboratory Science, Section I: Hematology Schmidt RM Ed. Vol. 3 pp. 15-31 CRC Press Boca Raton Florida). During the blood coagulation process, prothrombin is activated by cleavage of the first factor Xa site to form prethrombin-2, which is then cleaved at the second factor Xa site to form thrombin. Thrombin (M_r 34,000) consists of a 259 amino acid heavy chain and a 49 amino acid light chain connected by a single disulfide bond (Friezner Degen et al. 1983 Biochem 22:2087,DiBella et al. 1995 J Biol Chem 270:163).

However, all Gla-containing residues of prothrombin are cleaved during activation to thrombin. Human prothrombin (Jorgensen et al. 1987. J Biol Chem 262:6729) and thrombin (Russo et al. 1997. Prot Expr Purif 10:214) have both been successfully produced using recombinant Chinese hamster ovary (CHO) cell technology.

Factor XIII is the heterotetramer (a_2b_2) zymogen to factor $XIII_a$, which catalyzes the formation of intermolecular γ-glutamyl-ε-lysine bridges between fibrin molecules, which strengthens the clot against lysis (Lorand 1972. Ann NY Acad Sci 202:6). Earlier research has shown that the unglycosylated A-domain of factor XIII is sufficient for catalysis of the crosslinking of fibrin (Mary et al. 1988. Biochim Biophys Acta 966:328). Subsequently, functional factor XIII A-domain has been successfully produced in *E. coli* (Amann et al. 1988. Behring Inst Mitt 82:35, Lai et al. 1994. Prot Expr Purif 5:125). Although factor XIII is not currently used in therapeutic applications, it is being contemplated for future use as a wound closure aid.

General Overview of Transgenic Plants for Foreign Protein Production

Transgenic plants can be used for the production of high value, medicinally important proteins, for example, monoclonal antibodies (Hiatt et al. 1989. Nature 342:76, During et al. 1990. Plant Mol Biol 15:281, Benvenuto et al. 1991. Plant Mol Biol 17:865, Firek et al. 1993. Plant Mol Biol 23:861, Magnuson et al. 1996. Prot Expr Purif 7:220), human growth hormone (Kay et al. 1987. Science 236:1299), human serum albumin (Sijmons et al. 1990. Bio/Technol 8:217), human α–interferon (DeZoeten et al. 1989. Virology 172:213), and human erythropoietin (Matsumoto et al. 1995. Plant Mol Biol 27:1163). Unlike bacteria, plants perform many of the complex protein-processing steps required to produce mammalian proteins in an active form. Although most mammalian transgene products accumulate in plants at levels below 1% of soluble protein, Hiatt et al.

supra (1989) reported IgG antibody levels of up to 1.3% of soluble protein in tobacco. In addition, foreign protein levels of 14.4% soluble protein were reported in efforts to produce phytase, a digestive enzyme used in livestock, in transgenic tobacco (Pen et al. 1993. Bio/Technol 11:811-814). Despite these efforts, to our best knowledge, there have been no reports of the successful production of any human blood coagulation factor from transgenic plants.

Genetically engineered plant-based production of therapeutic proteins offers several advantages when compared to other production sources including human fluids/tissues, recombinant microbes, transfected animal cell lines or transgenic animals. First, farming of transgenic plants can significantly reduce the direct production cost of recombinant proteins. Large-scale agricultural systems may yield production costs as low as $6 to 60 per kg of raw protein product which are more than an order of magnitude less than similar direct production costs of recombinants in E.coli, currently estimated at $250 per kg (Kusnadi et al. 1997. Biotechnol Bioeng 56:473). Equally important, plant systems are capable of performing complex post-translational modifications necessary for activity in many human protein therapeutics. Signals for endomembrane targeting, signal peptide cleavage, BiP- or other chaperonin-mediated folding and oligomerization, N-linked glycosylation, isoprenylation, and sulfhydryl bridge formation are highly conserved between plants and animals (Chrispeels et al. 1991. Int Rev Cytol, 125:1, Bennett et al. 1991 *In* Plant Genetic Engineering, Grierson, Ed.: 199-237, Chapman and Hall. New York). In several reports, mammalian signal peptides were recognized *in planta*, leading to correct targeting of the plant endomembrane systems as well as correct assembly of protein subunits (Hiatt et al. supra 1989, Hein et al. 1991. Biotechnol Prog 7:455). Finally, plant-based recombinant proteins lead to increased product safety since plants do not serve as hosts for human or animal infectious agents (Cramer et al. 1996. Ann NY Acad Sci 792:62).

Plants may be transformed to express foreign DNA using a variety of methods including *Agrobacterium* transformation (An 1986. Plant Physiol 81:86, Hoekema et al. 1985. Plant Mol Biol 5:8589), microprojectile bombardment (Klein et al., Gene Transfer by Particle Bombardment, Plant Tissue Culture Manual, D1, p. 112, 1991, Kluwer Academic Publishers), pollen transformation (Saunders et al. 1997. U.S. Patent

5629183), chemical mediated uptake by protoplasts (Krens et al. 1982. Nature 296:72), and electroporation (Langridge et al. 1985 Plant Cell Reports 4:355).

Agrobacterium transformation

A. tumefaciens is the etiological agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms (DeCleene et al. 1976. Bot Rev 42:389). Virulent strains of *A. tumefaciens* contain large, tumor-inducing (Ti) plasmids (at about 200 kb). When wounded plants or plant tissues are cocultivated with such *Agrobacterium* strains, a portion of the Ti plasmid, called T-DNA, is transferred to and integrated into the nuclear genome of the infected plant cells (Hernalsteens et al. 1980. Nature 287:654, Lichtenstein et al. 1987. Genet Eng 6:104). The T-DNA encoded genes are transcribed and translated in the plant tissues, resulting in auxin, cytokinin and opine synthesis. The elevated auxin and cytokinin levels cause rapid plant cell proliferation, resulting in gall formation (Gheysen et al. 1985. DNA flux across genetic barriers: the crown gall phenomenon, In: Genetic Flux in Plants, Hohn et al., Eds., Springer, Wein, pp. 11-49).

To exploit Ti plasmids for genetic transformation, the native T-DNA sequence, responsible for gall formation, can be replaced with foreign DNA, including selectable markers as well as the gene of interest. The only components required for DNA transfer in the T-DNA region are the left and right border sequences (Zambryski et al. 1982. J Mol Appl Genet 1:361). However, since Ti plasmids are difficult to manipulate directly in vitro due to their large size, simplified systems have been developed. The most advanced method utilizes a binary vector system in which the binary vector contains the minimum elements required *in cis* (An supra 1986, An 1987. Meth Enzymol 153:292). Other functions necessary for the gene transfer mechanism are donated from a separate helper, Ti plasmid. The binary vector may be directly manipulated in an *E. coli* host and transferred to *A. tumefaciens* containing the helper Ti plasmid, through biparental or triparental mating. Finally, the T-DNA region is integrated into the plant nuclear genome by cocultivation of plant material with transformed *A. tumefaciens*. Representative tissues that have been transformed using an Agrobacterium method include tobacco (Barton et al. 1983. Cell 32:1033), tomato (Fillatti et al. 1987. Bio/Technol 5:726), sunflower (Everett et al. 1987. Bio/Technol 5:1201), cotton (Umbeck et al. 1987.

Bio/Technol 5:263), rapeseed (Pua et al. 1987. Bio/Technol 5:815), potato (Facciotti et al. 1985. Bio/Technol 3:241), poplar (Pythoud et al. 1987. Bio/Technol 5:1323) and soybean (Hinchee et al. 1988. Bio/Technol 6:915).

Microprojectile bombardment

In this method, gold or tungsten DNA-coated particles are accelerated towards target plant cells (Klein et al. 1987. Nature 327:70). This technique has been used to obtain stably transformed cultures of maize and tobacco (Klein et al. 1988. Bio/Technol 6:559) as well as for transient expression in onion. A comprehensive summary of microprojectile bombardment is given in Klein et al. Gene Transfer by Particle Bombardment, Plant Tissue Culture Manual. D1, p. 112, 1991, Kluwer Academic Publishers.

Pollen transformation

In pollen transformation techniques, plant germplasm is transformed with foreign DNA by introducing the DNA into pollen grains by techniques such as electroporation (Mishra et al. 1987. Plant Sci 52:135), mating ova of the desired plant line with the transformed pollen, and selecting for the transformed germplasm. The germinating pollen, resulting seed, and the progeny can each be screened for expression of the foreign gene. The transformed pollen can be used as a vector for introducing the foreign DNA into plant lines of similar or dissimilar origin, including both monocots and dicots. To date, pollen collected from tobacco and corn plants has been stably transformed via electroporation (Saunders et al. supra 1997). In addition, tobacco plants have been stably transformed to produce β-glucoronidase by mating ova with transformed tobacco pollen.

Chemical mediated uptake by protoplasts

DNA uptake via chemical stimulation was developed for the direct transformation of both monocot and dicot protoplasts (Krens et al. supra 1982). In this method, the plant cell wall is first degraded enzymatically to form protoplasts, using standard techniques. The protoplasts and vector are then incubated in the presence of polyethylene glycol, which facilitates transformation via direct insertion. Either a direct gene transfer vector or a Ti plasmid may be used in transformation. Since protoplast transformation can

facilitate measurable gene expression within 24 to 48 hours, this method has been used widely by many researchers (Prols et al. 1988. Plant Cell Reports 7:221, Topfer et al. 1988. Plant Cell Reports 7:225).

Electroporation

Introduction of DNA into plant protoplasts by treatment of the protoplasts with an electric pulse in the presence of the appropriate DNA is a process called electroporation (Fromm et al. 1985. Proc Natl Acad Sci USA 82:5824). Supercoiled or circular plasmid DNA is added to a suspension of protoplasts. The solution is mixed and subjected to a pulse of approximately 400 V/cm at room temperature for less than 10 to 100 □sec. A reversible physical breakdown of the membrane occurs to permit DNA uptake into the protoplasts. The success of the electroporation method is dependent, in part, on optimizing parameters relative to the membrane, the DNA and the electric field. Evidence for the success of transformation after electroporation has been measured by incorporation of radioactively labeled DNA (Tsong et al. 1985. Biblio Haematol 51:108), transient gene expression (Potter et al. 1984. Proc Natl Acad Sci USA 81:7161, Smithies et al. 1985. Nature 317:230), and the formation of stable transformants (Riggs et al. 1986. Proc Natl Acad Sci USA 83:5602, Stopper et al. 1985. Z Naturforsch 40:929). Due to rapid performance associated with electroporation, this technique is often applied for quick isolation and characterization of plant promoters and cis-acting elements (An et al. 1993. Techniques for isolating and characterizing plant transcription promoters, enhancers, and promoters. In: Methods in Plant Molecular Biology and Biotechnology, Glick et al., Eds., CRC Press, Boca Raton, pp. 155-165).

Plant (material) regeneration methods

After transformation of plant tissues, plants must be regenerated for characterization of stable transformants, selection, breeding and segregation analysis, and foreign protein (blood coagulation factor) production. Plants may be regenerated from a variety of tissues including callus culture, protoplasts, and *A. tumefaciens* tissues (i.e., explants or calli). Regeneration from callus tissue has been demonstrated in monocots,

such as corn, rice, barley, wheat and rye and dicots, such as sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from protoplasts is particularly useful for tissues transformed via direct gene transfer methods including electroporation, PEG-mediated transformation, or microparticle bombardment. Regeneration of plants from protoplasts has been demonstrated for rice (Abdulah et al. 1987. Bio/Technol 4:1987), tobacco (Potrykus et al. 1985. Mol Gen Genet 199:169), rapeseed (Kansha et al. 1986. Plant Cell Reports 5:101), and potato (Tavazza et al. 1986. Plant Cell Reports 5:243), among others.

Regeneration of plants from tissue transformed with *A. tumefaciens* has been demonstrated for several species of plants including tobacco (Horsch et al. 1985. Science 225:1229, Hererra-Estrella et al. 1983. Nature 303:209), sunflower (Everett et al. supra 1987), tomato (Fillatti et al. supra 1987), rapeseed (Pua et al. supra 1987), and cotton (Umbeck et al. supra 1987), among others.

SUMMARY OF THE INVENTION

The present invention is directed to transgenic plants that contain DNA sequences encoding for human coagulation factors. We have surprisingly discovered that correctly processed, active blood coagulation factors can be produced *in planta*. The present invention is further directed to compositions in transgenic plants or plant cell culture wherein the human coagulation factor is produced either as a protein having a human coagulation like procoagulant or coagulant activity or as an amino acid sequence substantially equivalent to that of a human coagulation factor.

These transgenic plant compositions are useful for producing viral free, active human-like blood coagulation proteins necessary for use in blood factor replacement (hemophilia), wound healing or other therapeutic applications contemplated for human blood coagulation factors. Viral pathogenicity is a major problem for blood-derived therapeutics because current production methods rely on human serum fractionation or transgenic mammalian cell culture methods. Both of these mammalian based systems can harbor pathogenic viruses resulting in contamination of derived therapeutic proteins with human pathogens. Plants systems are not known to harbor or transmit any human or mammalian viruses.

The transgenic plants are produced by transforming plants using known plant molecular biology methods, constructing vectors containing a DNA sequence or sequences encoding for human coagulation factors. It is to be understood that a reference herein to "human factor", "coagulation factor" or "coagulation factor N" (where N may include, but not be limited to, VIII, IX, XIII and thrombin) may include a reference to any of these, or to any combination of these. The scope of this specification should not be deemed limited to this list, but to any similar factor performing the equivalent function in the human body, and any derivative of such factor. Transgenic plants are useful for the production of human-like coagulation factors because they can perform necessary posttranslational modifications (e.g. glycosylation, folding and peptide cleavage) of these blood factor proteins by natural processing mechanisms, through further genetic engineering modifications, and/or through in vitro processing. These posttranslational modifications are important for activity, stability and clearance properties of these human-like coagulation factors.

The subject matter of the present invention is particularly pointed out and distinctly claimed in the concluding portion of this specification. However, the organization, compositions and methods of operation, together with further advantages and objects thereof, may best be understood by reference to the following description taken in connection with accompanying drawings wherein like reference characters refer to like elements.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 depicts schematically the blood coagulation cascade including intrinsic, extrinsic and common pathways. Figure reproduced from "Blood Coagulation," by Bithell in *The Normal Hematopoietic System* (Bloom and Thomas, Eds., Churchill, Livingston, N.Y., 1981, p. 579).

FIG. E1-1 depicts schematically the construction of pSP64-FVIIIc, a vector used to obtain the encoding sequence for pre-coagulation factor VIII for cloning into the plant expression vector pGA748. Pre-coagulation factor VIII cDNA encoding the 2332 amino acid mature protein plus the 19 amino acid native signal was inserted into the pSP64 plasmid at the Sal I site using a Sal I adapter, resulting in the new 10200 bp pSP64-

FVIIIc plasmid. This plasmid carried the ampicillin resistance gene for positive transformant selection in *E. coli*.

FIG. E1-2 depicts schematically the construction of pZD201, the plant expression vector carrying the pre-coagulation factor VIII encoding region. The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restriction enzyme site Xho I located between the CaMV 35S promoter and T7-T5 transcript terminator of binary vector pGA748, forming the 18800 bp plasmid pZD201. This plasmid carried the tetracycline resistance gene for positive transformant selection in *E. coli* and *Agrobacterium tumefaciens* as well as the kanamycin resistance gene for selection of whole plant and plant cell culture positive transformants.

FIG. E1-3 depicts schematically the construction of pGA2020, a vector used to obtain the encoding sequence for coagulation factor XIII A-domain for cloning into the plant expression vector pGA643. Coagulation factor XIII A-domain cDNA encoding the 731 amino acid protein and 29 amino acid signal peptide was inserted into pBluescript SK- at the Pst I site to adapt the Xba I site at the 5' end and the Cla I site at the 3'end, resulting in the new 5.3 kb pGA2020 plasmid. This plasmid carried the ampicillin resistance gene for positive transformant selection in *E. coli*.

FIG. E1-4 depicts schematically the construction of pGA2023, the plant expression vector carrying the coagulation factor XIII A-domain encoding region. The full length coagulation factor XIII A-domain cDNA was excised at Xba I/Cla I restriction sites and sequentially ligated into the compatible Xba I and Cla I restriction enzyme sites located between the CaMV 35S promoter and T7-T5 transcript terminator of binary vector pGA643, forming the 14.0 kb plasmid pGA2023. This plasmid carried the tetracycline resistance gene for positive transformant selection in *E. coli* and *A. tumefaciens* as well as the kanamycin resistance gene for selection of whole plant and plant cell culture positive transformants.

FIG. E1-5 is a construction map of plasmid vector pGA2042 containing the prethrombin-2 (PT2) gene.

FIG. E1-6 is a construction map of the binary vector pGA2043 containing the human prethrombin-2 gene.

FIG. E1-7 is a construction map of the plasmid vector pGA2049 containing the human prethrombin-2 gene.

FIG. E1-8 is a construction map of plasmid vector pGA2029 for the expression of human factor IX in transgenic plant.

FIG. E1-9 is a construction map of plasmid vector pGA2030 for the expression of human factor IX in transgenic plant.

FIG. E3-1 shows the result of dot blot immunoassay for T0 Factor VIII plant transformants. Positive control plasma-derived factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2. Negative control leaf protein extracted from untransformed *Nicotiana tabacum* cv. SR1 is shown as SR. Positive plant primary transformants (leaf protein extracts) are shown as 1004-3, 1006-2 and 1006-3.

FIG. E3-2 shows protein gel bands resulting from Western blot immunoassay completed on protein extracts from several plant transformants as compared to a non-transformed control culture and plasma-derived factor VIII standard (American Diagnostica, Greenwich, CT). Lane 1 shows the plasma-derived factor VIII (FVIIIc) standard; lane 2 shows total protein extracts from leaf explants taken from an untransformed *N. tabacum* cv. SR1 control; lanes 3 through 9 show total protein extracts from leaf explants taken from T1 plant lines derived from primary transformant tobacco plants.

FIG. E4-1 is a reverse electrophoresis gel image of PCR products of transgenic plant genomic DNA samples and control DNA samples.

FIG. E4-2 is a western blot analysis image of transgenic factor XIII A-subunit protein samples and various control protein samples.

FIG. E4-3 is a western blot analysis image of various transgenic factor XIII A-subunit protein samples and control protein samples.

FIG. E4-4 is a western blot analysis image of factor XIII A-subunit expression at different leaf positions.

FIG. E5-1 is a reverse electrophoresis gel image of the PCR products of prethrombin-2 transgenic plant genomic DNA samples and control DNA.

FIG. E5-2 is a western blot image of transgenic prethrombin-2 plant protein samples.

FIG. E5-3 is a silver stain analysis of protein samples purified by metal chelating sepharose column.

FIG. E5-4 is a construction map of transient expression plasmid vector pGA2054a.

FIG. E5-5 is a construction map of plasmid vector pGA2056 containing prothrombin gene.

FIG. E5-6 is a construction map of plasmid vector pGA2057 containing prethrombin-2 gene.

FIG. E5-7 is a construction map of plasmid vector pGA2058 for prothrombin transient expression.

FIG. E5-8 is a construction map of plasmid vector pGA2059 for prethrombin-2 transient expression.

FIG. E6-1 is a reverse electrophoresis gel image of the PCR products of factor IX transgenic plant genomic DNA samples transformed by pGA2029 and control DNA samples.

FIG. E6-2 is a reverse electrophoresis gel image of the PCR products of factor IX transgenic plant genomic DNA samples transformed by pGA2030 and control DNA samples.

FIG. E6-3 is an image of the protein gel Coomassie blue stain analysis of purified transgenic factor IX plant protein samples and control sample.

FIG. E8-1 is the construction map of transient expression plasmid vector pGA2052a.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes (a), plant, seeds and plant tissue capable of expressing human and human-like blood coagulation factors; (b) compositions capable of eliciting an activation response in human blood to induce clotting pathways upon

administration; (c) compositions useful for blood factor replacement, wound healing or other therapeutic applications contemplated for human blood coagulation factors; (d) methods for producing virus-free, human-like coagulation factors; (e) unique vectors containing DNA sequences encoding for blood coagulation factors and (f) methods for producing proteins having human and human-like procoagulant or coagulant activity or an amino acid sequence substantially that of a human coagulation factor in transgenic plants. This invention was based on the discovery that plant cells may be genetically transformed to produce human factor VIII in sufficient quantities to conduct biological testing and prove biological functionality. Similarly, it is also possible to transform plants to produce other human coagulation factors, such as but not limited to factors IX, XIII and thrombin. After having validated appropriate biological functionality, it is possible to genetically manipulate transgenic plants to produce commercially practicable quantities of coagulation factors. This invention is directed to these associated embodiments in all respects.

Specific Blood Coagulation Factors

Coagulation Factor VIII

Factor VIII is the zymogen of a large glycoprotein that functions in the blood coagulation cascade as a cofactor, functioning with calcium ions and phospholipid, in the factor IX_a-mediated activation of factor X in the intrinsic coagulation pathway. Factor VIII can be activated proteolytically by several coagulation enzymes, including thrombin.

Factor VIII is in chronically short supply, therefore making its use as a hemophilia A therapy highly cost prohibitive. In addition, resulting pharmaceutical products derived from plasma are highly impure, with a specific activity of 0.5 to 2 factor VIII units per milligram protein (one unit of factor VIII activity is by definition the activity present in one milliliter of normal plasma). Resulting plasma derived factor VIII purity is typically lower than 1% by weight (Wood et al. supra 1984). The high level of impurities result in a variety of serious complications including transmission of hepatitis A, B, and C, human parvovirus and human immunodeficiency virus (HIV) pathogens.

Factor VIII, at a relative mass of 300kD, is a heterodimeric molecule consisting of heavy chain and light chain peptide segments ionically held by a divalent cation,

presumed to be Ca^{2+}. The full-length protein coding sequence is recovered from a genomic library enriched for the human X chromosome by Wood et al. supra (1984). This sequence predicted a polypeptide of 2,351 amino acids, including a 19 amino acid signal peptide. The mature protein, at 2,332 amino acids, possesses a molecular weight of 330kD, which includes glycosylation, as determined by SDS polyacrylamide gel electrophoresis. Bare peptide molecular weight is calculated at approximately 265kD. Internal homologies were found which predicted a domain structure for the factor VIII protein consisting of a triplicated A-domain, a unique B-domain, and a duplicated C-domain arranged as follows: A1-A2-B-A3-C1-C2 (Gitschier et al. 1984. Nature 312:326, Toole et al. supra 1984). The unique B-domain contains 19 of the 25 potential asparagine linked glycosylation sites and corresponds to the portion of factor VIII which is unnecessary for procoagulant or coagulant activity. Factor VIII undergoes extensive post-translational processing in vivo including N- and O-linked glycosylation, cleavage of the primary translational protein after arginine at residue 1648 to yield light and heavy chains, and metal ion association of light and heavy chains (Kaufman et al. supra 1988).

To circumvent difficulties with viral pathogen transmission associated with human plasma-derived factor VIII and to lower product cost, factor VIII has been successfully expressed in a variety of mammalian cell culture systems. Initially, factor VIII was expressed in baby hamster kidney (BHK) cell lines, using the calcium phosphate coprecipitation method (Simonsen et al. supra 1983, Wigler et al. supra 1979) for integration of the 7kb protein encoding region (Wood et al. supra 1984, Capon et al. supra 1997). In this particular study, resulting factor VIII levels, determined through epitope screening, increased by 300-fold as compared to control T-cell hybridomas. Significant activation of factor IX_a determined using the Coatest assay was seen in the transfected BHK cells. Active recombinant human factor VIII has also been produced in Chinese hamster ovary (CHO) cells (Kaufman et al. supra 1988) and monkey COS7 cells (Toole et al. supra 1984, Truett et al. supra 1985). However, production of factor VIII using these mammalian cell lines does not completely eliminate the potential for transmission of human pathogens. Consequently, these cell lines will require in vivo and in vitro virus testing as well as mycoplasma detection during biosafety trials.

Beyond initial mammalian cell culture production studies, several modifications of recombinant factor VIII have been made at the molecular level to increase expression levels as well as reduce undesired immune responses in patients. Deletion of the B-domain of cDNA encoding factor VIII has been shown to increase expression in genetically modified Chinese Hamster Ovary (CHO) cells without affecting biological activity of the foreign peptide (Pittman et al. 1993. Blood 81:2925). Similarly, secretion of factor VIII in mammalian cell culture systems was increased 10-fold by replacing a carboxyterminal 110 amino acid sequence of the A1 domain with a homologous sequence from the factor V A1 domain (Marquette et al. 1995. J Biol Chem 270:10297). The replaced region is clustered with multiple short peptide sequences that have potential to bind BiP, thus inhibiting secretion. Also, the addition of von Willebrand Factor may be necessary for correct assembly of heavy and light domains of factor VIII and subsequent biological activity of the construct (Wise et al. 1991. J Biol Chem 266:21948). Von Willebrand Factor is a large (220 kDa) glycoprotein of complex multimeric structure which has been shown to stabilize factor VIII in vitro in mammalian cell culture applications (Kaufman et al. supra 1988).

In addition, it was found that the A2 and C2 domains of factor VIII contained the epitopes targeted by most inhibitory allo and autoantibodies (Lubin et al. 1994. J Biol Chem 269:8639). Since human inhibitors usually display limited or no reaction with porcine factor VIII, recombinant human/porcine factor VIII molecules were constructed by replacing the putative human A2 domain sequence (residues 387-604) with the homologous porcine sequence. This hybrid maintained full activity in the presence of A2 domain epitope specific antibodies but was inactivated in the presence of anti-C2 antibodies.

Outside the research reported in this document, production of factor VIII in recombinant hosts other than mammalian cells has not yet been successfully completed. Although factor VIII does not require γ–carboxylation of glutamic acid residues for activity, factor VIII does possess 25 separate glycosylation sites, which may preclude the use of prokaryotic host organisms for its production, especially if glycosylation is necessary for activity. In addition, the large (7.3 kb) protein coding region may preclude the use of many prokaryotic and lower eukaryotic hosts.

Coagulation Factor IX

Factor IX or Christmas Factor is the zymogen of a serine protease involved in the intrinsic coagulation pathway and specifically activates Factor X in the presence of Factor $VIII_a$, phospholipid, and calcium ion. Factor IX is used therapeutically for Hemophilia B (specific deficiency of human factor IX), which affects greater than 10,000 patients worldwide. Similar to those in factor VIII, impurities in plasma-derived factor IX concentrates may result in a variety of serious complications including transmission of hepatitis A, B, and C, human parvovirus and human immunodeficiency virus (HIV) pathogens.

The mature protein, at approximately 56kD, contains 416 amino acids, including 12 γ-carboxyglutamic acid (Gla) residues near the amino terminus and one β-hydroxyaspartic acid (Hya) residue at position 64. Vitamin K-dependent γ-carboxylation of at least a portion of these glutamic acid residues is necessary for factor IX activity. The Gla residues confer metal binding properties to factor IX, enabling two sequential conformational changes that are essential for the expression of membrane binding properties and coagulant activity (Borowski et al. 1986. J Biol Chem 261:14969, Liebman et al. 1985. Thromb Haemost 54:226). β-hydroxylation of aspartic acid at residue 64 does not appear to be necessary for factor IX procoagulant function (Derian et al. 1989. J Biol Chem 264:6615). Activation of factor IX is catalyzed by factor XI_a as well as several other serine proteases and results in an activation peptide (35 amino acids), and a light-chain (145 amino acids)/heavy-chain (236 amino acids) complex, linked by disulfide bonds (Kurachi et al. 1982. Proc Natl Acad Sci USA 79:6461).

Recombinant human factor IX with partial activity has been produced in human hepatoma cells (De la Salle et al. 1985. Nature 316:268), Chinese hamster ovary (CHO) cells (Kaufman et al. 1986. J Biol Chem 261:9622), and baby hamster kidney (BHK) cells (Busby et al. 1985. Nature 316:271). CHO cell-derived recombinant Factor IX required vitamin K to achieve γ-carboxylation in an average of 6.5 of 12 glutamic acid residues. At this level of γ-carboxylation, recombinant factor IX possessed about 50% of the specific activity of plasma-derived, fully carboxylated factor IX. Some partially carboxylated forms of factor IX are likely to be partially active if only non-essential γ-

carboxyglutamic acid residues are missing (Kaufman et al. supra 1986). γ-carboxylation of factor IX has also been achieved in vitro using a partially purified carboxylating enzyme system from bovine liver. Use of this in vitro system boosted apparent carboxylation levels from 3 to 8 residues per factor IX molecule (Soute et al. 1989. Thromb Haemost 61:238).

Coagulation Factor XIII

Factor XIII functions after vascular injury to catalyze the formation of γ-glutamyl-ε-lysyl covalent bonds between fibrin molecules, thereby strengthening the forming fibrin clot. Because of this clot strengthening ability, exogenous factor XIII has been contemplated as a wound healing therapeutic, although it is not currently approved for this type of use. Structurally, plasma-borne factor XIII is a tetramer composed of two A-chains (81kD) and two B-chains (75kD) (Lai et al. supra 1994). Factor XIII derived from platelets and placenta consists of an A-chain homodimer, only. The A-chains possess the catalytic domain of factor XIII and appear to be unglycosylated and non-disulfide bonded. The B-chains appear to prevent □-thrombin mediated proteolytic inactivation of factor $XIII_a$ in plasma (Mary et al. supra 1988). Factor XIII activation is initiated by thrombin cleavage of the 4 kD activation peptide from the N-terminus of A-chains. B-chains, when present, subsequently undergo calcium-dependent dissociation from the rest of the molecule.

Initial efforts in *E. coli*-based production of recombinant factor XIII A-chains yielded an inactive product that was of proper size as well as immunoreactive to anti-factor XIII antibodies (Amann et al. supra 1988). In subsequent research, active A-chains were expressed in the JM 105 strain of *E. coli* (Lai et al. supra 1994). Resulting protein behaved as a dimer on gel filtration analysis, was thrombin- and calcium-activated, cross-linked fibrin and bound to fibrin to the same extent as purified plasma factor XIII.

Thrombin

Thrombin is a serine protease (M_r=34kD) that converts fibrinogen to fibrin by limited proteolysis, releasing fibrinopeptides A and B in the final stages of the common

clotting pathway. Fibrin creates a crosslinked matrix (clot) that leads to hemostasis. Thrombin is currently used therapeutically for topical hemostasis.

The corresponding zymogen to thrombin, prothrombin (M_r=72kD), synthesized in the liver, is converted to thrombin by minor proteolysis by factor X_a in the intrinsic pathway, in the presence of factor V_a, calcium ion, and phospholipid (Friezner Degen et al. supra 1983). In the extrinsic pathway, prothrombin is activated through interaction with factor VII_a and tissue factor (Nemerson et al. 1985. Thrombosis Res. 40:351). Like factor VII, prothrombin contains 10 γ–carboxyglutamic acid residues within the first forty amino acids from the N-terminus. These residues are required for attachment of prothrombin to prothrombinase and are removed with the reaction peptide during prothrombin activation. γ-carboxylation is accomplished by membrane-bound vitamin K-dependent carboxylase (MacGillivray et al. 1984. Biochem, 23:1626).

Human prothrombin cDNA has been expressed in CHO cells in the presence of vitamin K, yielding a fully γ-carboxylated protein with specific coagulant activity equivalent to that of plasma derived prothrombin (Jorgensen et al. supra 1987). In related research, prethrombin-2, an intermediate in prothrombin activation, was cloned into CHO cells (Russo et al. supra 1997) as well as *E. coli* (DiBella et al. supra 1995). Prethrombin-2 lacks the N-terminal pro-sequence of prothrombin, but has not yet been cleaved to form distinct thrombin light and heavy chains. The resulting recombinant prethrombin-2 was proteolytically activated by ecarin, a protease derived from *Echis carinatus* snake venom. The *E. coli*-derived prethrombin-2 also required refolding prior to activation.

Plant Transformation Vectors

The vectors in the present invention contain DNA coding for specific blood coagulation factors and are capable of transforming plants. Foreign DNA is DNA that is exogenous to or not naturally found in the organism to be transformed. Foreign DNA can be inserted into cloning vectors to transform plants and is derived from or has substantial sequence homology to DNA encoding specific blood coagulation factors. Plant expression vectors in this invention are produced using standard molecular cloning procedures (Ausubel et al. 1992. Current protocols in molecular biology. Wiley, New

York) and splicing PCR techniques (Marks et al. 1992. J Biol Chem 267:16007). However, the vector produced will depend on which type of transformation and which species of plant is being transformed. A Ti-plasmid-derived vector is appropriate for Agrobacterium-mediated transformation of both whole plants and plant cell culture. In electroporation of plant protoplasts, a Ti-plasmid or another appropriate direct gene transfer vector may be used to accomplish transformation. If specific plant tissues are to be transformed, an appropriate vector that is active in these tissues must be used. Specific examples of plant expression vectors are given by Hoekema et al. (supra 1985), Mushegian et al. (1995. Microbiol Rev 59:548) and An (supra 1987).

The construction of vectors can be accomplished using an *E. coli* host, such as MC1000, among others. These vectors may be used directly for direct gene transfer techniques such as protoplast electroporation or micro-injection. If an Agrobacterium-mediated transformation technique is to be used, the vector must first be transferred to a suitable strain, such as *A. tumefaciens* LBA4404, among others. The vector may be transferred from *E. coli* to the Agrobacterium using the "freeze-thaw" technique (An et al. 1988. In: Plant Molecular Biology Manual A3, pp. 1-19, Kluwer Academic, Dordrecht), among others.

The vectors of the present invention contain DNA sequences encoding blood coagulation factors as well as blood coagulation factor-like molecules with coagulant or procoagulant activity. DNA sequences may consist of full-length cDNA or genomic clones, which encode intact blood coagulation factors, such as the 7.2 kb full-length cDNA clone for coagulation factor VIII encoding for the full length, mature 2,332 amino acid protein, among others. In contrast, DNA sequences may consist of specific fragments of encoding sequences for blood coagulation factors, such as the fragment of 2.0 kb prothrombin cDNA that encodes prethrombin-2, the direct precursor to thrombin (Russo et al. supra 1997) or the fragment of factor VIII cDNA that encodes B-domain deleted factor VIII (Pittman et al. supra 1993), among others. In addition, DNA sequences may consist of hybrid constructs of encoding regions of two or more different blood coagulation factors such as the factor V/factor VIII hybrid construct in which 330 bp of the A1 domain of factor VIII was replaced with a homologous region of factor V (Marquette et al. supra 1995).

Specific genetic regulatory elements (i.e., transcriptional promoters, transcriptional terminators, signal peptide encode regions, untranslated leader sequences) are inserted into the vectors, in this invention, to yield desired expression characteristics. The transcriptional promoter, which is necessary to initiate transcription, is placed at the upstream or 5' end of the ligation site of the human blood coagulation factor encoding sequence. Specific examples of transcriptional promoters include the CaMV 35S promoter which generally affords constitutive expression throughout plant tissues (Odell et al. 1985. Nature 313:810) and the ribulose bis-phosphate carboxylase small subunit gene promoter which limits expression of the foreign gene to tissues possessing chloroplasts (i.e., green tissues, such as the leaf and stem) (Pichersky et al. 1986. Proc Natl Acad Sci USA 83:3880), among others. The transcriptional terminator, which is necessary to terminate transcription, is inserted at the downstream or 3' end of the said ligation site. Specific examples include the *A. tumefaciens* Ti plasmid nopaline synthase (T_{nos}) and the mannopine synthase (T_{mas}) transcription terminators, among others. Further, an additional regulatory element encoding a signal peptide may be added between the promoter and the 5' end of the ligation site or between the 3' end of the ligation site and the terminator in order to relegate the product human blood coagulation factor to a specific cellular organelle. Specific examples of signal peptides include the ribulose bis-phosphate carboxylase small subunit signal peptide which affords chloroplast localization (Fritz et al. 1993. Gene 137: 271), the tobacco PR-1 protein signal peptide which affords apoplast localization (Cornellissen et al. 1986. Nature 321:531) and the C-terminal KDEL coding region which affords endoplasmic reticulum localization (Pelham 1990. Trends Biochem Sci 15:483), among others. In addition, untranslated leader sequences (UTL) may be added either between the promoter and the additional regulatory element encoding the signal peptide or at the 3' end of the encoding sequence to obtain greater mRNA stability between transcription and translation events. Specific examples include the 36bp UTL of the alfalfa mosaic virus subgenomic RNA 4 (Jobling et al. 1987. Nature, 325:622) and the ribulose bis-phosphate carboxylase small subunit UTL (Fritz et al. supra 1993).

Plant Transformation

The cells of plants are transformed with the vectors described previously by any technique known in the art, including those described in the references discussed previously and those to follow. These techniques include but are not limited to the Agrobacterium method in which Ti-plasmid bearing *A. tumefaciens* is cocultivated with plant tissue as described by Hoekema et al. supra 1985. Other suitable transformation techniques include electroporation (Langridge et al. supra 1985), chemical mediated uptake by protoplasts (Krens et al. supra 1982), microinjection (Crossway et al. 1986. Mol Gen Genet 303:179), microprojectile bombardment (Klein et al. supra 1991), and pollen transformation (Saunders et al. supra 1997).

Following transformation, the transformed plant tissue is selected by conventional techniques, such as antibiotic resistance screening. Positively transformed tissues (containing the foreign gene) may be regenerated using techniques known in the art including shoot/root regeneration or suspension cell cultivation, among others. Regenerated plants and/or suspension cell cultures are screened to confirm gene insertion, transcription, foreign protein accumulation and foreign protein activity. Progeny of the regenerated plants are similarly screened to develop improved plant and seed lines. The foreign gene can be moved into other genetic lines using a variety of techniques, including classical breeding, protoplast fusion, nuclear transfer, and chromosome transfer.

Activity of Blood Coagulation Compositions

As with other transgenic plant production of pharmaceutical preparations, validation of successful cloning and expression of human coagulation factors in transgenic plants and plant cell cultures must be established. All blood coagulation factors derived from plant material should demonstrate immunological cross-reactivity of antibodies raised against plasma-derived blood coagulation factor with clone-derived blood coagulation factor as validated by enzyme-linked immunosorbent assay. In addition, all blood coagulation factors derived from plant material should possess comparable relative protein size with respective plasma-derived coagulation factors.

Functional plant derived blood coagulation proteins should specifically perform proper activation function in the coagulation cascade within in vitro assays. A variety of available assays, generally based on conversion of a synthetic, chromogenic substrate, will test the following:

Factor VIII: Activation of factor X in the presence of factor IX_a, calcium ion, and phospholipid following factor VIII activation in the presence of thrombin.

Factor IX: Activation of factor X in the presence of factor $VIII_a$, calcium ion, and phospholipid following factor IX activation in the presence of calcium ion and factor XI_a.

Factor XIII: Transglutaminase activity (i.e., γ -glutamyl- ε -lysyl crosslinking of fibrin) following factor XIII activation in the presence of thrombin.

Thrombin: Release of fibrinopeptide A during the conversion of fibrinogen to soluble fibrin in the presence of calcium ions.

In addition, activity of factors VIII and IX may be tested through the correction of factor VIII-deficient and factor IX-deficient plasma samples, respectively. Factor VIII can also be tested for binding and subsequent elution from immobilized von Willebrand's factor.

Example 1- Vector Construction

Vectors useful for transforming plants with DNA sequences encoding for human coagulation factors are pZD201, pGA2023, pGA2049, pGA 2029 and pGA2030. Sufficient information has been provided in Example 1 in order to enable the construction of these vectors from starting materials and methods that are widely known and generally available. The information available herein will enable the construction of similar vectors from other starting materials.

A. Construction of Plasmid Vector pZD201 (Factor VIII)

The *E. coli* plasmid pSP64-FVIIIc (ATCC No. 39812) containing the gene encoding the full-length polypeptide of factor VIII cDNA, derived from human fetal liver, was obtained from ATCC (**FIG. E1-1**). Coagulation factor VIII is the 2332 amino acid protein that contains a heavy chain, B-domain and a light chain. The 7.2kb pre-

coagulation Factor VIII cDNA encodes native signal peptide (the first 19 amino acid at the NH_2-termini) as well as the mature protein.

The full length pre-coagulation factor VIII cDNA was excised with Sal I restriction enzyme and sequentially ligated into the compatible restriction enzyme site Xho I located between the CaMV 35S promoter and T7-T5 transcription terminator of the binary vector pGA748, forming the 18.8 kb plasmid pZD201 (**FIG. E1-2**).

B. Construction of Plasmid Vector pGA2023 (Factor XIII A-domain)

The pUC18-FXIII vector containing the gene (cDNA) encoding the full-length polypeptide of coagulation factor XIII A-domain, derived from adult human uterus (pregnant), is obtained from Earl W. Davie, University of Washington, Seattle, WA (Ichinose et al. 1986. Biochem 25:6900). Coagulation factor XIII is a tetramer composed of two A subunits linked as a dimer and two loosely associated B subunits (Schwartz et al. 1973. J Biol Chem 248:1395). The A subunit consists of 731 amino acid residues with a molecular weight of 83,150. The B subunit polypeptide is composed of 641 amino acid residues with a molecular weight of 73,183 and contains a carbohydrate component that adds significantly to the molecular weight of the circulating protein. During coagulation, the A subunit zymogen is activated by the thrombin catalyzed cleavage of an amino terminal peptide (4 kDa). The activated A subunits catalyze the formation of γ-glutamyl-ε-lysine peptide bonds, facilitating the crosslinking of fibrin and thereby strengthening the formed fibrin clot (Lorand et al. 1980. Prog Haemost Thromb 5:245). The 3.9kb pre-coagulation factor XIII A domain cDNA encodes the mature protein preceded by the native signal peptide.

The full length pre-coagulation factor XIII A-domain cDNA is excised from pUC18-FXIII with a Pst I restriction enzyme and sequentially ligated into the compatible restriction enzyme site Pst I located on the multiple cloning site on pBluescript SK- (Strategene, La Jolla, CA) as shown in **FIG. E1-3**. Subsequently, the factor XIII A-domain clone is excised from pGA2020 at Xba I/Cla I restriction sites and ligated into compatible Xba I and Cla I restriction sites, respectively, located between the CaMV 35S promoter and T7-T5 transcription terminator of the binary vector pGA643, forming the 14.0 kb plasmid pGA2023 (**FIG. E1-4**).

C. Construction of Plasmid Vectors pGA 2043 and pGA2049 (Thrombin)

The vector pHII-3 containing the cDNA encoding the full-length polypeptide of prothrombin may be obtained from Earl W. Davie of University of Washington, Seattle, WA, (Friezner Degen et al. supra 1983). Prothrombin (M_r 72,000) is a vitamin K dependent protein that participates in the final phase of blood coagulation. During the blood coagulation process, prothrombin may be activated by cleavage at the first factor X_a site to form prethrombin-2. Prethrombin-2 is subsequently cleaved at the second factor X_a site to form thrombin. Correctly processed thrombin (M_r 34,000) consists of a 259 amino acid heavy chain and a 49 amino acid light chain connected by a single disulfide bond.

The cloning of the thrombin expression vector consisted of several steps. In the first step, the native thrombin signal peptide sequence (36 amino acids) was cloned using the full-length human prothrombin gene as the DNA template and the following primers. The forward primer was designed to adapt a restriction enzyme site Xba I and an initiation codon (ATG) at the 5' end of the signal peptide sequence, and the 3' end sequence of the signal peptide was used for the reverse primer. The PCR signal peptide sequence contained 111 bp counting from the adapted Xba I site.

Forward primer (P1):

5'-GCA TGC TCT AGA ATG CAG CTG CCT GGC TGC CTG GCC CTG GCT-3'

Reverse primer (P2):

5'- TCG CCG GAC CCG CTG GAG CAG-3'

Second, another PCR reaction was conducted using the purified PCR product to add a 12-bp sequence of the prethrombin-2 gene to the signal sequence using the purified PCR product from the first step as the DNA template, the P1 primer and the following reverse primer. The reverse primer was designed using the P2 primer sequence flanked with a 12-bp prethrombin-2 sequence starting at the first factor Xa codon site. The PCR

product was designated as prothrombin SP. The PCR product sequence had a length of 123 bp counting from the adapted Xba I site.

Reverse primer (P5):

5'- CTC ACT TGT GGC GGT TCG CCG GAC CCG CTG GAG CAG -3'

Subsequently, the prethrombin-2 gene without signal peptide sequence (PT2 w/o SP) was cloned at the first factor Xa codon using the prothrombin gene as the DNA template and the following primers. The forward primer was designed directly downstream of the first factor Xa codon site of the 5' end of the prethrombin-2 gene and the reverse primer was designed to adapt a 6-histidine tag sequence $(His)_6$ followed by a stop codon (TGA) and a restriction enzyme site Cla I at the 3' end. The 6-histidine peptide at the C-terminus can be used for purification of expressed prethrombin-2 protein.

Forward primer (P3):

5'-ACC GCC ACA AGT GAG TAC CAG-3'

Reverse primer (P4):

5'- GCA TGC ATC GAT CTA ATG ATG ATG ATG ATG ATG CTC TCC AAA CTG ATC AAT GAC CTT CTG -3'

Finally, the prothrombin SP sequence and the prethrombin-2 gene sequence were attached via PCR using the purified PCR products, 123 SP and PT2 w/o SP as the DNA templates, and the primers P1 and P4. The PCR product had 1062 bp and was designated as PT2, containing restriction enzymes sites, Xba I at the 5' end and Cla I at the 3' end. Resulting purified PCR PT2 DNA was cut with Xba I and Cla I and consequently cloned into Xba I and Cla I restriction enzyme sites of the pBluescript SK- vector (Strategene, La Jolla, CA) to form vector pGA2042 (4.02 kb) as shown in **FIG. E1-5**. The constructed prethrombin-2 gene with the native signal peptide sequence was excised using Cla I and Xba I and sequentially ligated into the compatible restriction enzyme sites located between the Rubisco (RbcS-3C) promoter and T5-T7 transcription

terminator of a binary expression vector pZD261 (13.4 kb), forming the 14.5 kb vector pGA2043 as shown in **FIG. E1-6**, where BL is the T-DNA left border; BR is the T-DNA right border; npt is the neomycin phosphotransferase gene; oriT is the RK2 origin of conjugal transfer; oriV is the RK2 origin of replication; pRBC/AMV is the Rubisco (RbcS-3C) promoter with a alfalfa mosaic virus leader sequence; T5 is the transcription terminator 5; T7 is the transcription terminator 7; tet is the tetracycline resistance gene of RK2 plasmid; and trfA* is the segment for a replication protein.

In addition, the prethrombin-2 gene with native signal sequence was also cloned into another binary expression vector pGA643 (11.7 kb), forming the 12.8 kb vector pGA2049 as shown in **FIG. E1-7**. The expression of the prethrombin-2 gene in the binary vector pGA2049 was under the control of the 35S promoter (P35S) and T5-T7 terminator. These constructed binary vectors were directly transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (An supra 1987).

D. Construction of Plasmid Vector pGA2029 and pGA2030 (Factor IX)

The pGA748-F9c vector containing the gene (cDNA) encoding the full-length polypeptide of pre-coagulation factor IX, derived from human liver, is obtained from Dr. Earl Davie, Biochemistry Department, University of Washington, Seattle, Washington (Kurachi et al. supra 1982). Coagulation factor IX is a single chain, 416 amino acid glycoprotein with a molecular weight of approximately 56,000 and contains 12 γ-carboxyglutamic acid (gla) residues in the amino-terminal region. During the coagulation process, factor IX is converted to factor IX_a by factor XI_a. Factor IX_a then converts factor X to factor X_a in the presence of factor $VIII_a$, phospholipid, and Ca^{2+}. At least a portion of the 12 N-terminal gla residues are necessary for factor IX activity. The 1.5kb coagulation factor IX cDNA encodes the mature protein, an 18 amino acid pro-sequence and a 28 amino acid signal peptide.

The full length pre-pro-coagulation factor IX cDNA was cloned by PCR using the primers below. The forward primer was designed to adapt the Xba I restriction enzyme site at the 5' end of the factor IX cDNA coding for the mature factor IX protein and the reverse primer was designed to adapt a 6-histidine tag sequence $(His)_6$ followed by a stop codon (TAG) and a restriction enzyme site Cla I at the 3' end. The 6-histidine peptide at

the C-terminus can be used for the purification of expressed pre-pro-factor IX after expression.

Forward primer (P1):

5'-C TGC TCT AGA TAT AAT TCA GGT AAA TTG GAA G-3'

Reverse primer (P2):

5'-T AGA AGA TCT TTA ATG ATG ATG ATG ATG ATG AGT GAG CTT TGT TTT TTC CTT AAT CCA GTT GAC-3'

After PCR and digestion with restriction enzymes Xba I and Cla I, pre-pro-factor IX DNA (1281 bp) was ligated into compatible restriction enzyme sites located between the Rubisco (RbcS-3C) promoter (Sugita et al. 1987 Mol. Gen. Genet. 209:247) and T5-T7 transcription terminator of binary expression vectors, pZD256 and pZD261 (13.4 kb), forming the 14.8 kb vectors pGA2029 and pGA2030 as shown in **FIG.'s E1-8, E1-9,** respectively, where BL is the T-DNA left border; BR is the T-DNA right border; npt is the neomycin phosphotransferase gene; oriT is the RK2 origin of conjugal transfer; oriV is the RK2 origin of replication; pRBC/AMV is the RbcS-3C promoter with an alfalfa mosaic virus leader sequence; pRBC/VSP is the RbcC-3C promoter with a vacuole signal sequence T5 is the transcription terminator 5; T7 is the transcription terminator 7; tet is the tetracycline resistance gene of RK2 plasmid; and trfA* is the segment for a replication protein. Expression of pre-pro-factor IX is under the control of the RbcS-3C promoter with a vacuole signal peptide sequence in pGA2029 and with an alfalfa mosaic virus (AMV) leader sequence in pGA2030. These constructed binary vectors were directly transferred into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (An supra 1987).

Example 2- Agrobacterium Mediated Transformation

N. tabacum plants were transformed by *A. tumefaciens* LBA4404 containing the expression vectors for factors VIII, IX, XIII-A domain and thrombin production prepared according to Example 1. These plasmids were directly transferred into *A. tumefaciens* LBA4404 using the freeze-thaw method (An supra 1988). Subsequent plant

transformation was accomplished by co-cultivation of tobacco leaf disks or calli with the Agrobacterium. After 2 to 3 days, explant tissues were removed to fresh medium containing the antibiotics carbenicillin to kill the Agrobacterium and kanamycin to select positive transformants. Whole plants were regenerated on shoot and root regeneration media containing kanamycin.

Example 3 - Factor VIII Production

A. Protein Immunoblotting

Vector pZD201 described in Example 1A is used. The tobacco plants are transformed using the Agrobacterium method described in Example 2. After obtaining positive transformants via kanamycin resistance screening, mature tobacco plants and calli are assayed for the presence of the human coagulation factor VIII. Preliminary protein immunoblotting (dot blot assays-**FIG. E3-1**) completed using extractable leaf protein showed the presence of coagulation factor VIII antigen in the leaf tissues of T0 whole plant transformants. As seen in **FIG. E3-1**, strong factor VIII:ag expression characteristics were seen in plants 1004-3, 1006-2 and 1006-3. Positive control factor VIII standards (American Diagnostica, Greenwich, CT) are shown as S1 and S2 whereas negative control leaf protein derived from untransformed *N. tabacum* cv. SR1 is shown as SR. After completion of the dot-blot immunoassay, T0 plants were self-pollenated, resulting in T1 seedstock. T1 seeds were subsequently germinated on kanamycin selective media and mature plants were grown in a controlled environment. Western immunoblot assays shown on **FIG. E3-2**, completed on leaf protein extracts of T1 plants regenerated from various T0 plant lines, indicate the presence of immunoreactive bands which appear to be comparable in size to those of plasma-derived factor VIII (American Diagnostica, Greenwich, CT). The predominant band appears at approximately 240 kD. Additional faint bands from plasma derived factor VIII and plant transformants appear to correspond to factor VIII heavy chain at 90-200 kD and light chain at approximately 80 kD. The appearance of comparable immunoreactive bands between plant-derived and plasma-derived human factor VIII suggest that plant-derived factor VIII undergoes correct, human-like post-translational modifications. Sheep anti-human factor VIII:C polyclonal antibody (Haematologic Technologies, Inc., Essex Jct., VT) and sheep anti-

human factor VIII polyclonal antibody AB787 (Chemicon International, Inc., Temecula, CA) were mixed and used for both the Western blot and dot blot immunoassays.

B. Factor VIII Activity Assay

The transgenic plant leaf material was harvested and total soluble protein was extracted using standard techniques. Functionality of recombinant human factor VIII was analyzed using the Coatest method (Helena Laboratories, Beaumont, Texas). After activation by thrombin, factor VIII acts as a cofactor in the conversion of factor X to factor X_a by factor IX_a when calcium and phospholipid are present. In the Coatest assay, the quantity of factor X_a generated was determined using a specific chromogenic substrate (MeO-CO-D-CHG-Gly-Arg-pNa) and was directly proportional to the amount of factor VIII in the sample tested. During the functional analysis, total protein samples from plants expressing factor VIII protein as well as untransformed control plants and CBHI cellulase expressing tobacco plants were tested.

Results of the Coatest assay are shown in Table E3-1. In each individual sample, 1.5 mg of soluble plant protein was used. Tests A, B and C were completed on separate days and each required a separate untransformed plant control. In tests A and B, the duration of incubation after factor X_a addition was 5 minutes. In test C, the duration of incubation after factor X_a addition was 4 minutes. Also in test C, aliquots of factor VIII reference plasma standard (Helena Laboratories, Beaumont, TX) were added to two separate tobacco plant controls. Results from tests A and B, as compared to increases in absorbance mediated by the addition of factor VIII in test C, clearly show the presence of factor VIII procoagulant activity in tobacco plant lines 1005-5, 1005-6, and 1006-3. The highest level of activity observed in line 1006-3 would roughly correspond to 26 ng of factor VIII per 1500 µg sample (based on linear regression of calibration data from test C) or an expression level of 0.002% of extractable leaf protein. This surprising result indicates that recombinant human factor VIII is correctly processed *in planta* resulting in procoagulant activity.

Table E3-1 – Results of Coatest Assay for selected plant transformants

Test	Plant Line	Change in Absorbance Upon addition of Factor X_a (ΔA_{405})	Change in Absorbance Compared to Plant Control ΔA_{405}[sample] - ΔA_{405}[control])
A	1005-5	0.322	0.118
A	1005-6	0.269	0.065
A	plant control	0.204	-
B	1006-3	0.676	0.239
B	plant control	0.437	-
C	plant control 1 w/ 5 ng FVIII	0.134	0.106
C	plant control 1 w/ 10 ng FVIII	0.176	0.148
C	plant control 1 w/ 30 ng FVIII	0.268	0.24
C	plant control 1	0.028	-
C	plant control 2 w/ 5 ng FVIII	0.177	0.137
C	plant control 2 w/ 10 ng FVIII	0.222	0.182
C	plant control 2 w/ 30 ng FVIII	0.305	0.265
C	plant control 2	0.040	-

Example 4- Factor XIII A-Domain

A. Factor XIII Gene Insertion Confirmation.

Vector pGA2023 described in Example 1 is used. The tobacco plants were transformed using the Agrobacterium method described in Example 2. After obtaining positive transformants via kanamycin resistance screening, fifty tobacco plant transformants were obtained through selective media screening and used to confirm the insertion of the Factor XIII gene in the transgenic plant genome. Genomic DNA of these plants was prepared using a hexadecyltrimethyl ammonium bromide (CTAB) mini-preparation method. Briefly in this method, a small amount of plant material (25-100 mg) was ground into a fine powder with liquid nitrogen and extracted at 65°C for genomic DNA in an extraction buffer containing 3% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 5 mM ascorbic acid, and 2% (w/v) polyvinypyrolidone (PVP-40; Sigma Chemical Co.), subsequently, a phenol-chloroform extraction was conducted. The upper aqueous phase was saved after centrifugation at 10,000 × g for five minutes and genomic DNA was precipitated in isopropanol at the presence of sodium acetate.

The resulting DNA samples were used for factor XIII gene amplification by PCR using the following primers derived from the factor XIII gene. The forward primer is specific to the sequence immediately downstream the initiation codon (ATG) and the reverse primer is specific to sequence immediately upstream of the stop codon (TGA).

Forward primer:

5'-ACT TCC AGG ACC GCC TTT GGA GGC AGA AGA-3'

Reverse primer:

5'-GGA AGG TCG TCT TTG AAT CTG CAC GTC CAG-3'

PCR results are shown in **FIG. E4-1**, where lane S is the size marker, lane SR1 is the non-transformed plant as the negative control, lane FXIII is the factor XIII A-subunit DNA as the positive control, and lanes 11, 23, 33, 35, 45, 47 are transgenic plant samples. Results show that a DNA band of 2.3 kb is present for all shown transgenic plants, matching up with the PCR band of factor XIII DNA. No PCR 2.3 kb band is observed in the non-transformed plant DNA sample. The results here confirm insertion of the factor XIII gene into the tobacco plant genome. A total of fifty transformed plants were screened for factor XIII gene insertion and forty plants showed factor XIII A subunit gene insertion.

B. Western Blot Analysis.

A high salt buffer (HSB), which contains 50 mM pH 7.5 Tris-HCl, 0.5 M NaCl, 0.05% Nonidet P-40 and 1.0 mM phenylmethyl sulfonyfluoride (PMSF) was used for total protein extraction. Leaf materials of the transgenic plants grown in square culturing vessels were ground and extracted in the HSB in an eppendorf tube using an eppendorf tube grinder. Plant extract supernatants were collected after centrifugation at 20,000 × g for 10 min. Total protein was determined using a Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples containing 100 µg total protein were denatured at 95°C for 5 minutes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subsequently analyzed by a 4-20 % gradient SDS-PAGE gel (Bio-Rad Laboratories, Richmond, CA). An amount of 3.65 µg human plasma coagulation factor XIII was used in SDS-PAGE as a positive control (Haematologic Technologies Inc., Essex Junction, VT). The separated protein was

electrophoretically blotted onto a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Western blot was carried out with sheep anti-human factor XIII polyclonal antibody PAHFXIII-S used at a 1:1000 dilution as the primary antibody (Haematologic Technologies Inc., Essex Junction, VT) and the alkaline phosphatase-conjugated rabbit anti-sheep IgG used at a 1:1000 dilution as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The immune complexes were detected using an Immun-Blot Colorimetric Assay Kit (Bio-Rad Laboratories, Hercules, CA).

The Western blot results of transgenic factor XIII plants are shown in **FIG. E4-2** and **FIG. E4-3**. In **FIG. E4-2**, two transgenic plant protein samples (lanes 1 and 2) were used in the Western blot analysis while three control protein samples were used as the negative controls, C1: non-transformed plant protein sample; C2 and C3: transgenic plant protein samples transformed with a binary expression vector containing no factor XIII gene. Results indicate that the 83 kDa factor XIII bands are expressed only in the transgenic factor XIII plants but not in the non-transformed control plant and transformed control plants. **FIG. E4-3** shows the results of another Western blot analysis for various factor XIII transgenic plants, where lane FXIII is human plasma coagulation factor XIII as the positive control, lane SR1 is the non-transformed plant as the negative control, and lanes 11, 23, 33, 35, 45, 47 are the transgenic plant samples. The Western blots reveal at least two major proteins. One of the protein bands is the major 83 kDa protein and has the identical size as the human plasma factor XIII. The other protein band has a size of 210 kDa which may be a complex of unprocessed, dimerized factor XIII A subunit protein. A total of forty transgenic plants were screened using Western blot analysis and thirty seven out of forty plants expressed the human factor XIII A-subunit. The expression yield based on the Western blot analysis ranged approximately from 0.1 to 1.8% of the total extracted soluble leaf protein.

C. Activity Analysis of Plant Derived Human Factor XIII A Subunit.

Plant protein samples were extracted from plant leaf materials in a buffer containing 50 mM pH 7.6 mM Tris-HCl . The extraction mixture was centrifuged at 4°C at 20,000 × g for 15 minutes and the supernatant was collected and used for both protein

and factor XIII activity assay. The activity of plant derived human factor XIII was measured using a method described by Hornyak et al. (1989 Biochem 28:7326). Factor XIII A subunit activity is defined as the amount of monodansylcadaverine incorporated into casein by the transamidase activity of activated factor XIII A-subunit (Coggan et al 1984 Anal Biochem 137:402). Monodansylcadaverine incorporation into casein is measured as the fluorescence increase in the reaction mixture. Before the fluorescence incorporation reaction, factor XIII A-subunit is activated by the thrombin catalyzed cleavage of an amino terminal peptide (4 kDa). In this method, 100 µl extracted plant protein sample was added to a 2.0 ml reaction tube, along with 100 µl of 50 mM pH 7.6 Tris-HCl buffer, 20 µl of 4 mM monodansylcadaverine prepared in 50 mM Tris-HCl buffer, 50 µl 0.4 M $CaCl_2$ prepared in 50 mM Tris-HCl buffer, 1200 µl 50 mM, pH 9.0 Bicine buffer. The reaction mixture was warmed in a 37°C water bath for one minute. Reactions with non-transformed plant protein sample, factor XIII standard (3.65 µg) and blank (200 µl Tris-HCl buffer) were also set up as controls. 50 µl of α-thrombin solution was added to the above reaction mixture and incubated in a 37°C water bath for 10 minutes to activate factor XIII. The α-thrombin reagent (500 unit/ml, Haematologic Technologies Inc., Essex Junction, VT) was prepared in 25 mM pH 7.5 Tris-HCl buffer and 25% glycerol. After the above incubation, 50 µl 0.2 M DTT solution was added to the reaction mixture, which was incubated at 37°C for one minute. 200 µl 2% N,N'-dimethylcasein was added to the reaction mixture and the time zero fluorescence was measured in a fluorometer. Consequently, the reaction mixture was further incubated in a 37°C water batch to monitor the increase of fluorescence. The net increase in fluorescence was used for the calculation of factor XIII activity. Table E4-1 shows the activity of plant derived factor XIII A-subunit using human plasma factor XIII as the positive control and the non-transformed SR1 plant protein sample as the negative. Pure human plasma factor XIII has an activity of 50.2 unit per milligram of total protein. Results show that the activity of plant recombinant factor XIII A subunit ranges from 0.012 to 0.237 unit per milligram of soluble protein, which is equivalent to 0.1 to 2.5% factor XIII A-subunit protein of total plant soluble protein.

TABLE E4-1

Sample	Total protein (μg)	*Delta Fluorescence	Less Fluorescence	FXIII Activity (unit)	FXIII Specific Activity (unit/mg protein)
FXIII STD	3.65	1949	1886	0.183	50.2
Blank	0	63	-	-	-
SR1	230	104	-	-	-
#33	190	568	464	0.0450	0.237
#35	150	250	146	0.0142	0.095
#38	250	237	133	0.0129	0.052
#45	220	169	65	0.0063	0.029
#47	280	159	55	0.0053	0.019
#48	280	151	47	0.0046	0.016
#49	270	137	33	0.0032	0.012

*Delta fluorescence was calculated by subtracting the fluorescence at 270 minutes by the fluorescence at time zero.

D. Expression of Plant Recombinant Factor XIII A Subunit at Various Leaf Positions.

Transgenic tobacco plants expressing human factor XIII A-subunit protein were cultivated to maturity in the greenhouse. The expression of factor XIII A subunit was tested at different leaf positions using Western blot analysis. Protein samples were extracted using HSB buffer from leaves at positions 1, 3, 5, and 7 counted from top to bottom excluding necrotic yellow leaves. Protein samples were analyzed using 7.5 % SDS-PAGE and subsequently blotted onto a nitrocellulose membrane. Western blot was carried out with sheep anti-human factor XIII polyclonal antibody PAHFXIII-S used at 1:1000 dilution as the primary antibody (Haematologic Technologies Inc.) and the alkaline phosphatase-conjugated rabbit anti-sheep IgG used at 1:1000 dilution as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The immune

complexes were detected using an Immun-Blot Colorimetric Assay Kit (Bio-Rad Laboratories). The results of the Western blot analysis are shown in **FIG. E4-4**, where lane FXIII is the human factor XIII protein sample (3.65 μg) as the positive control, lane SR1 is the non-transformed plant protein sample (100 μg), lane SJ is a transgenic plant protein sample (100 μg) from the top leaf grown in a square culturing vessel, and lanes G1, G3, G5, and G7 are the protein samples (100 μg) from leaf positions 1, 3, 5, and 7, respectively from the transgenic plant. It can be seen that the expression of factor XIII A-subunit has similar expression levels at different leaf positions. In addition, the expression level of the factor XIII protein is the same between the transgenic plant from a square culturing vessel and the plant grown in a greenhouse. However, the immuno-active 210 kDa protein band vanishes gradually as the leaf position changes from 1 to 3, 5, and 7. This is probably due to the completion of post-translational modification of the presumed factor XIII A-subunit complex, releasing single chains of factor XIII A-subunit.

Example 5- Thrombin

A. Prethrombin-2 Gene Insertion Confirmation.

Vectors pGA2043 and pGA2049 described in Example 1 were used. The tobacco plants were transformed using the Agrobacterium method described in Example 2. After obtaining positive transformants via kanamycin resistance screening, mature tobacco seventeen tobacco plant transformants were obtained through the selective media screening process and used to confirm the insertion of the prethrombin-2 gene in the transgenic plant genome. Genomic DNA of these plants was prepared using a hexadecyltrimethyl ammonium bromide (CTAB) mini-preparation method. Briefly in this method, a small amount of plant material (25-100 mg) was ground into fine power with liquid nitrogen and extracted at 65°C for genomic DNA in an extraction buffer containing 3% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 5 mM Ascorbic acid, and 2% (w/v) polyvinypyrolidone (PVP-40; Sigma Chemical Co.); subsequently, a phenol-chloroform extraction was conducted. The upper aqueous phase was saved after centrifugation at 10,000 × g for five minutes and genomic DNA was precipitated in isopropanol in the presence of sodium acetate. The resulting DNA samples were used for

prethrombin-2 gene amplification by PCR using the previously described primers P1 and P4. PCR results are shown in **FIG. E5-1**, where lane S is the size marker, lane PT is the prethrombin-2 DNA with the signal sequence as the positive control, lane SR1 is the non-transformed plant as the negative control, lanes 1, 2, 3 are the transgenic plant samples transformed with the binary vector pGA2043, and lane 4 is the transgenic plant sample transformed with the binary vector pGA2049. Results show that a distinct DNA band of 1.06 kb is present for all the transgenic plants, matching up with the PCR band of the prethrombin-2 gene with the signal sequence. No 1.06 kb band is observed in the non-transformed plant DNA sample. The results here confirm insertion of the prethrombin-2 gene into the tobacco plant genome.

B. Western Blot Analysis.

Total plant leaf protein was extracted from transformed and non-transformed plants using a high salt buffer (HSB), which contains 50 mM pH 7.5 Tris-HCl, 0.5 M NaCl, 0.05% Nonidet P-40 and 1.0 mM phenylmethyl sulfonyfluoride (PMSF). Leaf materials of the transgenic plants grown in square culturing vessels were ground and extracted in the HSB in an eppendorf tube using an eppendorf tube grinder. Plant extract supernatant samples were collected after centrifugation at 20,000 × g for 10 min. Total protein was determined using a Bradford Reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as the protein standard. Protein samples containing 100 μg total protein were denatured at 95°C for 5 minutes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subsequently analyzed by a 4-20 % gradient SDS-PAGE (Bio-Rad Laboratōries, Richmond, CA). An amount of 6.5 μg human plasma α-thrombin was used in the SDS-PAGE as the positive control (Haematologic Technologies Inc., Essex Junction, VT). The separated protein was electrophoretically blotted onto a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Western blotting was carried out with sheep anti-human prothrombin polyclonal antibody PAHFII-S used at a 1:1000 dilution as the primary antibody (Haematologic Technologies Inc., Essex Junction, VT) and alkaline phosphatase-conjugated rabbit anti-sheep IgG used at a 1:1000 dilution as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The immune

complexes were detected using an Immun-Blot Colorimetric Assay Kit (Bio-Rad Laboratories, Hercules, CA). The Western blot results of transgenic prethrombin-2 plants are shown in **FIG. E5-2**, where lane TH is the human plasma α–thrombin served as the positive control, and lanes 1, 2, 3, and 4 are the prethrombin-2 transgenic plant samples, lane C is the non-transformed plant served as the negative control. The transgenic samples 3 and 4 show a 39 kDa prethrombin-2 band, which is about 5 kDa larger than human alpha-thrombin (34 kDa). The 5-kDa difference is probably due to incomplete cleavage of the signal peptide (4.5 kDa) and the 6-histidine tag (0.66 kDa).

C. Purification and Silver Stain Analysis of Prethrombin-2 Protein.

Total protein samples were extracted from the leaf materials of transgenic prethrombin-2 plants using HSB buffer supplemented with 1 mM PMSF. One gram of leaf tissue was first ground into power in liquid nitrogen and extracted in 5 ml of HSB buffer using a mortar and a pestle. The sample was subsequently centrifuged at 20,000 × g for 10 min and the supernatant was saved for the following protein purification. Metal chelating *Sepharose Fast Flow* (Pharmacia Biotech, Piscataway, NJ) was used for the prethrombin-2 protein purification. For each sample, a 3-ml disposable column was filled with one milliliter of metal chelating sepharose . The sepharose was prepared as follows: wash with four volumes of deionized water, activate with two volume of 0.1 M $NiSO_4$ solution, wash again with six volumes of deionized water, and equilibrate with four volumes of the HSB buffer. Five milliliters of the protein sample supernatant was loaded into the column, which was consequently washed with six volumes of 10 mM pH 8.0 imidazole solution. The protein bound to the sepharose was eluted with 500 mM pH 8.0 imidazole solution and the eluted sample was collected in 0.5-ml aliquots. The second and the third 0.50 ml samples were combined since they contained most of the eluted protein based on the protein concentration analysis using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). Protein samples containing 10 μg eluted protein were denatured at 95°C in a SDS-PAGE sample buffer and subsequently analyzed by 4-20 % gradient SDS-PAGE. After separation, the gel was stained using a Silver Stain Plus kit (Bio-Rad Laboratories). The results are shown in **FIG. E5-3**, where lane SM is the protein size marker, lane TH is the human alpha-thrombin protein sample, lane C1 is the

non-transformed plant protein sample, lane C2 is the transgenic plant control protein sample, lanes 1, 2, and 3 are the prethrombin-2 transgenic plant protein samples. As seen in the **FIG. E5-3**, samples 2 and 3 indicate a 39 kDa protein band, the plant derived prethrombin-2 protein, which is identical to the one shown in **FIG. E5-2**. This band is not shown in either the non-transformed or transformed control samples.

D. Prothrombin and Prethrombin-2 Transient Expression Vector Construction.

The full-length human prothrombin gene (2006 bp) in vector pHII-3 was obtained from Dr. Earl W. Davie, Biochemistry Department, University of Washington, Seattle, Washington (Friezner Degen et al., supra 1983). Several steps were involved in constructing the prothrombin transient expression vector. In the first step, an expression cassette with only the cauliflower mosaic virus promoter CaMV 35S promoter (P 35S), the multiple cloning sites (MSC), and the transcription terminator (T7-T5) in a binary vector pGA643 (An, 1995) was cloned using PCR by Taq polymerase (Stratagene, La Jolla, CA) with the following primers.

Forward primer:
5'- CGA ACA CTT GAT ACA TGT GCC TGA GAA ATA -3'
Reverse primer:
5'-CTA TGA AGA TCG GCG GCA ATA GCT TCT TAG-3'

The cloned expression cassette was ligated into a plasmid vector pGEM-T (Promega, Madison, WI) to form a transient plant expression vector pGA2054a, as shown in **FIG. E5-4**. The vector pGA2054a also contains an ampicillin resistance gene (Amp) and a f1 phage origin (f1 ori). Foreign genes can be cloned into this transient vector pGA2054a for expression tests.

Second, human prothrombin and prethrombin genes were cloned using PCR by Taq polymerase (Stratagene) to adapt a Xba I cloning site at the 5' end of the gene and a Cla I cloning site at the 3' end. The following primers P1 and P3 were used for the prothrombin PCR cloning reaction and the primers P2 and P3 were used for the

prethrombin-2 cloning reaction. The cloned prethrombin-2 was also adapted with the initiation codon ATG at the 5' end of the gene.

Forward primer (P1):

5'-GCA TGC TCT AGA ATG CAG CTG CCT GGC TGC CTG GCC CTG GCT-3'

Forward primer (P2):

5'-GCA TGC TCT AGA ATG GCC ATC GAA GGG CGT ACC GCC ACA-3'

Reverse primer (P3):

5'- GCA TGC ATC GAT TTA CTC TCC AAA CTG ATC AAT GAC CTT CTG -3'

The cloned prothrombin gene was subsequently ligated into the vector pGEM-T (Promega, Madison, WI) to form the vector pGA2056 as shown in **FIG. E5-5**. Also, the prethrombin2 gene was ligated into pGEM-T to form the vector pGA2057 also as shown in **FIG. E5-6**. The prothrombin gene in pGA2056 and prethrombin-2 gene pGA2057 were, respectively, excised out with Xba I and Cla I restriction enzymes. Sequentially, these gene were cloned into the transient vector pGA2054a at the Xba I and Cla I sites, respectively, forming the prothrombin transient expression vector pGA2058 shown in **FIG. E5-7** and the prethrombin-2 transient expression vector pGA2059 shown in **FIG. E5-8**, respectively.

E. Transient Analysis Using Electroporation of Tobacco Protoplasts.

A 3-day old NT1 tobacco cell suspension was used for the preparation of protoplasts. Briefly, protoplasts were isolated by treating the suspension cells with a pH 5.8 solution containing 10 mg/l cellulase, 500 µg/ml pectoplyase (Kanematsu-Gosho, Los Angeles, CA) and 0.4 M D-mannitol at 28°C for 20 minutes at 100 rpm. The protoplasts were then extensively washed with 0.4 M mannitol to remove cellulase and pectolyase. Finally, 1×10^6 protoplasts were resuspended in 0.5 ml of pH 5.5 electroporation buffer containing 2.38 mg/ml HEPES, 8.76 mg/ml NaCl, 735 µg/ml CaCl2 and 0.4 M D-mannitol.

After addition of 20 μg supercoiled plasmid DNA of pGA2058 and pGA2059 and 10 μg salmon sperm DNA as carrier DNA, the protoplasts were then electroporated using a 300 volt pulse with 210 μF capacitor. The treated protoplasts were subsequently transferred into 7 ml of protoplast culture medium in a Petri dish and cultured for 48 hours at 28°C. The culture medium is a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 4.3 mg/ml MS salt supplemented with 3% sucrose, 0.18 mg/ml KH_2PO_4, 0.1 mg/ml inositol, 1 μg/ml thiamine hydrochloride, and 0.2 μg/ml 2.4-dichlorophenoxyacetic acid (2.4-D), and 0.4 M D-mannitol.

The cultured protoplasts were collected by gentle centrifugation and suspended in 100 μl extraction buffer containing 50 mM Tris-HCl pH 8.3, 227 mM NaCl, 1 mg/ml bovine serum albumin, and 1 mg/ml sodium azide. Protein samples were extracted by sonicating the protoplasts three times for 8 seconds at 30-second intervals. The protein samples were harvested by centrifuging the sonicated mixture at 15,000 g for 5 minutes. The supernatant was saved and protein concentration was measured using the Bio-Rad Protein Assay method (Bio-Rad, Hercules, CA).

Fifty micrograms of extracted protein was used to measure the α–thrombin activity released by either transiently expressed prothrombin or prethrombin-2. Human prothrombin (Haematologic Technologies Inc., Vermont) was used as a positive and the protein samples from electroporated non-transformed NT1 protoplasts were used as negative controls. Ecarin (Sigma) was used to cleave the prothrombin and prethrombin-2 to release the active α–thrombin protein. One unit of ecarin was added to each sample and the sample was incubated at 37°C for 15 minutes. For each analysis, the ecarin-reacted 100 μl sample was quickly mixed with 0.9 ml extraction buffer and 0.1 ml of 1.25 mg/ml Chromozym TH solution and the change of absorbance was monitored spectrophotometrically. The results are shown in Table E5-1.

Table E5-1 Alpha-thrombin activity released by Ecarin treatment in protein samples prepared by transient expression method.

No.	Samples*	Human Prothrombin Addition (ng)	Delta Absorbance at 405 nm between 0 and 24 hrs	Delta Absorbance between Sample No. 1	Calculated alpha-thrombin in the sample (pg)
1	NT1	0	0.075	-	-
2	NT1	2.7	1.185	1.11	-
3	NT1	27	1.828	1.753	-
4	NT1	135	1.804	1.729	-
5	prothrombin	0	0.116	0.041	100
6	prothrombin	0	0.042	Not active	-
7	prothrombin	0	0.102	0.027	66
8	prothrombin	0	0.082	0.007	17
9	prethrombin-2	0	0.061	Not active	-
10	prethrombin-2	0	0.127	0.052	126
11	prethrombin-2	0	0.174	0.099	240
12	prethrombin-2	0	0.076	Not active	-

*Each sample was equally treated with one unit of Ecarin. Transgenic samples were transiently expressed by individually prepared plasmid DNA.

Example 6- Pro-coagulation Factor IX

A. Factor IX Gene Insertion Confirmation.

Vectors pGA2029 and pGA2030 described in Example 1 are used. The tobacco plants are transformed using the Agrobacterium method described in Example 2. After obtaining positive transformants via kanamycin resistance screening, mature tobacco forty and thirty seven tobacco plant transformants, respectively for the transformation of pGA2029 and pGA2030, were obtained through the selective media screening process and used to confirm the insertion of the factor IX gene in the transgenic plant genome. Genomic DNA of these plants was prepared using a hexadecyltrimethyl ammonium bromide (CTAB) mini-preparation method. Briefly in this method, a small amount of plant material (25-100 mg) was ground into fine power with liquid nitrogen and extracted at 65°C for genomic DNA in an extraction buffer containing 3% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 5 mM ascorbic

acid, and 2% (w/v) polyvinypyrolidone (PVP-40; Sigma Chemical Co.). Subsequently, a phenol-chloroform extraction was conducted. The upper aqueous phase was saved after centrifugation at 10,000 × g for five minutes and genomic DNA was precipitated in isopropanol in the presence of sodium acetate. The resulting DNA samples were used for factor IX gene amplification by PCR using the previously described primers P1 and P2. PCR results are shown in **FIG. E6-1**, where lane S is the size marker, lane FIX is the factor IX DNA serving as a positive control, lane SR1 is the non-transformed plant serving as the negative control, and lanes 1, 2, 3, and 4 are transgenic plant samples transformed with the binary vector pGA2029. **FIG. E6-2** shows analogous results for the transgenic plants transformed with the binary vector pGA2030. Results in both **FIG. E6-1** and **FIG. E6-2** show that a distinct DNA band of 1.28 kb, corresponding to the size of the pre-pro-Factor IX gene (positive control), is present for all the transgenic plants. Factor IX gene insertion was confirmed in a total of thirty eight and thirty four transgenic plants for binary vectors pGA2029 and pGA2030, respectively.

B. Protein Purification and Protein Gel Staining.

Plant leaf materials were harvested from transformed and non-transformed plants. Total protein was extracted using a high salt buffer (HSB), which contains 50 mM pH 7.5 Tris-HCl, 0.5 M NaCl, 0.05% Nonidet P-40 and 1.0 mM phenylmethyl sulfonyfluoride (PMSF). Leaf materials were first ground to power in liquid nitrogen with a mortar and a pestle, and subsequently extracted in an equal volume of the HSB buffer. Plant extract supernatants were collected after centrifugation at 20,000 × g for 10 min. Total protein was determined using a Bradford Reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as protein standard. The protein extract was purified for the 6-histidine-tagged factor IX protein using metal chelating sepharose (Pharmacia Biotech, Piscataway, NJ). One milliliter of the metal chelating sepharose was loaded into a 3-ml size column and activated by 0.1 M $NiSO_4$ solution. After washed thoroughly with six volumes of deionized water and equilibrated with four volumes of the HSB buffer, the protein sample supernatant (5 ml) was loaded into the column, which was consequently washed with six volumes of 10 mM pH 8.0 imidazole solution. The protein bound to the sepharose was eluted with a 50-mM pH 6.0 imidazole solution and the eluted sample was

collected in 0.5-ml aliquots. The second 0.5-ml aliquot contained the most protein and was used for the gel electrophoresis. Protein samples containing 40 µg eluted protein were denatured at 95°C in a SDS-PAGE sample buffer and subsequently analyzed by 4-20 % gradient SDS-PAGE. After separation, the gel was stained using a Coommasie Brilliant blue solution. The results are shown in **FIG. E6-3**, where lane FIX is the factor IX standard (Haematologic Technologies Inc., Essex Junction, VT), lane C is a non-transformed plant protein sample, and lanes 1 and 2 are the transformed plant protein samples. Results indicate that the recombinant plant derived human factor IX has a size of about 65 kD. The transformed plant sample in lane 1 shows a unique band of about 58 kD, which is not observed in the non-transformed plant sample. The transformed plant sample in lane 2 shows no similar 58 kD protein band in the gel stain.

Example 7 - In Vitro Activation of Pro-factor IX

Purified pro-coagulation factor IX described in Example 6 is used. Acarboxyl-glutamyl residues in acarboxyl-pro-coagulation factor IX can be γ-carboxylated by treatment with vitamin K-dependent carboxylase (Soute et al. supra 1989). Vitamin K-dependent carboxylase is prepared from normal cow liver and partly purified as described by Soute et al. (1987. Thromb Haemostas 57:77). Standard γ-carboxylation reaction mixtures, as reported by Van Haarlem et al. (1987. FEBS Lett 222:353), consist of 1.0 mg partially purified carboxylase, 2 µg recombinant acarboxyl-pro-factor IX, 0.15 M NaCl, 1 M $(NH_4)_2SO_4$, 20 mM Tris/HCL pH 7.5, 5 µCi $NaH^{14}CO_3$, 8 mM $MnCl_2$, 0.16% (w/v), 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, 0.4 mg/mL phosphatidylcholine (added as mixed micelles with cholate in 1:1 [w/w] ratio), reducing agents (0.2 mM thioredoxin, 0.2 µM thioredoxin reductase, and 4 mM NADPH), and 0.4 mM vitamin K hydroquinone in a total reaction volume of 125 µL. Vitamin K hydroquinone is solubilized by mixing it with the phosphatidylcholine before preparing the mixed micelles according to the method of De Metz et al. (1981. J Biol Chem 256:10843). The presence of gla residues in pro-factor IX can be tested by completing the γ-carboxylation reaction using $NaH^{14}CO_3$ and measuring incorporation of $^{14}CO_2$ using scintillation counting. Alternatively, the presence of gla residues can be confirmed based on colorimetric detection using 4-diazobenzenesulfonic acid staining of polyacrylamide

gels as reported by Jie et al. supra (1995). Correctly γ-carboxylated pro-factor IX may be separated from the reaction mixture using standard protein purification methods.

To excise the 18 amino acid pro-peptide and release correctly processed coagulation factor IX, approximately 1mg of ammonium sulfate-precipitated protein from the γ-carboxylation reaction mixture is dissolved in a 1 mL reaction mixture containing 0.4M Tris-HCL (pH 7.0), 0.1% lubrol, 1 mM EDTA, 1 mg/mL pepstatin, 1 mg/mL bestatin, and 50 g of KEX2 endopeptidase in the presence of 2mM $CaCl_2$ at 37 °C for one hour (see U.S. Patent No. 5,234,830). After subsequent ammonium sulfate precipitation and resuspension of protein in Tris-HCl (pH 7.0) buffer, Western blot immunoassay can be used to determine the extent of cleavage of the N-terminal, 18 amino acid pro-peptide.

Factor IX coagulant activity is determined with a two-stage assay using factor IX-deficient plasma (Proctor et al. 1961. J Clin Pathol 36:212). One unit of factor IX activity represents the amount of factor IX in 1 mL of normal human pooled plasma.

Example 8 – Transient Expression of Human Coagulation Factor XIII A Subunit in Maize Protoplasts.

A. Factor XIII Transient Expression Vector Construction.

The expression cassette of factor XIII A subunit in the binary plasmid vector pGA2023 as shown in **FIG. E1-4** was used for the construction of a transient expression vector. The expression of factor XIII A subunit is under the control of the 35S promoter and transcription is terminated by the T7 terminator. The factor XIII expression cassette (3.6 kb) was cloned by PCR with Taq polymerase (Stratagene, La Jolla, CA) using the following primers:

Forward primer:

5'- CGA ACA CTT GAT ACA TGT GCC TGA GAA ATA -3'

Reverse primer:

5'-CTA TGA AGA TCG GCG GCA ATA GCT TCT TAG-3'

The cloned expression cassette was directly ligated into a plasmid vector pGEM-T (Promega, Madison, WI) to form a transient factor XIII expression vector pGA2052a, as shown in

FIG. E8-1. The vector pGA2052a also contains an ampicillin resistance gene (Amp) and a f1 phage origin (f1 ori).

B. Transient Analysis Using Electroporation of Maize Protoplasts.

Maize (*Zea mays*) protoplasts will be used for transient factor XIII expression analysis. Maize cell suspension culture is grown in a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 4.3 mg/ml MS salt supplemented with 3% sucrose, 0.18 mg/ml KH_2PO_4, 0.1 mg/ml inositol, 1 µg/ml thiamine hydrochloride, and 0.5 µg/ml 2.4-dichlorophenoxyacetic acid (2.4-D). A 4-day old maize cell suspension will be used for the preparation of protoplasts. Briefly, protoplasts are isolated by treating the suspension cells with a pH 5.8 solution containing 10 mg/l cellulase, 500 µg/ml pectoplyase (Kanematsu-Gosho, Los Angeles, CA) and 0.4 M D-mannitol at 28°C for 2 to 4 hours with gentle shaking at 100 rpm. The protoplasts are checked every 30 minutes to avoid overdigestion. Upon the completion of enzyme digestion, the protoplasts are extensively washed with 0.4 M mannitol to remove cellulase and pectolyase. Finally, 1-2×10^6 protoplasts are resuspended in 0.5 ml of pH 5.5 electroporation buffer containing 2.38 mg/ml HEPES, 8.76 mg ml NaCl, 735 µg/ml CaCl2 and 0.4 M D-mannitol.

After addition of 20 µg supercoil plasmid DNA of pGA2052a and 10 µg salmon sperm carrier DNA, the protoplasts can be electroporated using a 300 volt pulse with a 210 µF capacitor. The treated protoplasts are subsequently transferred in 7 ml of protoplast culture medium in a Petri dish and cultured for 48 hours at 28°C prior to protein extraction. The culture medium is modified MS medium with the addition of 0.4 M D-mannitol. After cultivation for 48 hours, the cultured protoplasts can be collected by gentle centrifugation and suspended in plant extraction buffer containing 50 mM pH 7.5 Tris-HCl, 0.5 M NaCl, 0.05% Nonidet P-40 and 1.0 mM phenylmethyl sulfonyfluoride (PMSF). Protein samples can be extracted by sonicating the protoplasts on ice three times for 8 seconds at 30-second intervals. The protein samples can be

harvested by centrifuging the sonicated mixture at 15,000 g for 5 minutes. Supernatant will retained used for protein concentration analyses, Western blot analyses and activity assays. The Western blot analysis will follow the same procedure outlined in previous tobacco-based factor XIII expression examples. Likewise, factor XIII A subunit activity will be assayed using the method described in the previous tobacco-based factor XIII expression example, where factor XIII A subunit activity is defined as the amount of monodansylcadaverine incorporated into casein by the transamidase activity of activated factor XIII A-subunit.

Closure

While preferred and alternative embodiments of the present invention have been shown and described, it will be apparent to those skilled in the art that many changes and modifications may be made without departing from the invention in its broader aspects. The appended claims are therefore intended to cover all such changes and modifications as fall within the true spirit and scope of the invention.

CLAIMS

We claim:

1. A coagulation factor capable of eliciting an activation response in the human blood clotting pathways, comprising a blood clotting protein derived from a transgenic plant, said transgenic plant comprising and expressing a DNA sequence coding for a blood clotting factor protein, said blood clotting protein extracted from said transgenic plant being capable of eliciting an activation response in a human blood coagulation pathway upon administration of the coagulation factor.

2. The coagulation factor of Claim 1, wherein the DNA sequence comprises a gene, a combination of genes, a gene fragment or combination of gene fragments coding for said coagulation factor.

3. The coagulation factor of Claim 1, wherein the coagulation factor comprises factor V, factor VII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, prethrombin 2, thrombin, fibrin, fibrinogen, tissue factor, von Willebrand factor, prekallikrein, HMW kininogen and combinations thereof

4. The coagulation factor of Claim 1, wherein the coagulation factor is selected from the group consisting of factor VIII, factor XIII, factor IX, prothrombin, prethrombin 2, thrombin and combinations thereof.

5. The coagulation factor of Claim 1, wherein the transgenic plant is selected from the group consisting essentially of dicotyledonous and monocotyledonous angiosperm plants.

6. A coagulation factor capable of eliciting an activation response in a human blood clotting pathway, comprising a blood clotting protein derived from a transgenic plant comprising and expressing a DNA sequence coding for a blood clotting factor protein, said blood clotting protein being free of human viral pathogens and

capable of eliciting an activation response in the human blood clotting pathway upon administration of the coagulation factor.

7. A transgenic plant comprising and expressing a DNA sequence coding for human coagulation factors, said coagulation factors being capable of eliciting an activation response in the human blood clotting pathway upon administration of the coagulation factor.

8. The transgenic plant of Claim 7, wherein the coagulation factor expressed therein is selected from the group consisting of factor V, factor VII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, prethrombin 2 thrombin, fibrin, fibrinogen, tissue factor, von Willebrand factor, prekallikrein, HMW kininogen and combinations thereof.

9. The transgenic plant of Claim 7, wherein the coagulation factor expressed therein comprises factor VIII, factor XIII, factor IX, prothrombin, prethrombin 2, thrombin and combinations thereof.

10. The transgenic plant of Claim 7, wherein the plant is selected from the group consisting essentially of monocotyledonous and dicotyledonous angiosperm plants.

11. A method of eliciting an activation response in human blood clotting pathways comprising:

(a) producing a transgenic plant comprising and expressing a DNA sequence coding for human coagulation factors capable of eliciting activation response in human blood clotting pathway;

(b) extracting said human coagulation factors from said transgenic plant;

(c) administering said human coagulation factor to a human patient in order to induce an activation response in said patient's blood clotting pathway.

12. The method of Claim 11, further comprising extracting a coagulation factor from said transgenic plant selected from the group consisting of factor V, factor VII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, prethrombin 2, thrombin, fibrin, fibrinogen, tissue factor, von Willebrand factor, prekallikrein, HMW kininogen and combinations thereof.

13. The method of Claim 12, further comprising extracting the coagulation factor from plant cells, said coagulation factor selected from the group consisting of factor VIII, factor XIII, factor IX, prothrombin, prethrombin 2, thrombin and combinations thereof.

14. A method of producing from a plant human like coagulation factors free of human viral pathogens, comprising:

(a) obtaining a positive transformant of the plant, the positive transformant carrying genetic material encoding the production of the human coagulation factor;

(b) reproducing the positive transformant; and

(c) extracting commercial quantities of the human coagulation factor from the plant.

15. The method of claim 14, wherein the coagulation factor is reproduced in whole plants.

16. The method of claim 14, wherein the coagulation factor is reproduced in a plant tissue culture.

17. The method of claim 14, wherein said human coagulation factor is a protein having a human coagulation factor-type procoagulant or coagulant activity.

18. The method as recited in claim 14, wherein obtaining the positive transformant comprises the step of modifying an encoding sequence for a human coagulation factor for subcloning into a plant expression vector.

19. The method as recited in claim 18, wherein said encoding sequence is selected from the group consisting of copy DNA, genomic DNA and combinations thereof.

20. The method as recited in claim 18, wherein the method further comprises:

(a) subcloning the encoding sequence into a plant expression vector and obtaining a subcloned plant expression vector;

(b) transferring the subcloned plant expression vector into a plurality of plant cells;

(c) selecting a plurality of positive transformants from the plurality of plant cells on an antibiotic selective media;

(d) inducing growth of the positive transformant in whole plants or suspensions; and

(e) extracting a quantity of the human coagulation factor from the plants of step (d).

21. The method as recited in claim 19, wherein transferring is by direct particle bombardment.

22. The method as recited in claim 19, wherein transferring is by Agrobacterium mediated transformation.

23. The method as recited in claim 18, wherein transferring is by pollen transformation.

24. The method as recited in claim 21, wherein the Agrobacterium mediated transformation comprises the steps of:

(a) placing the subcloned plant expression vector into an Agrobacterium; and

(b) cocultivating the Agrobacterium containing the subcloned plant expression vector with the plurality of plant cells.

25. The method as recited in claim 19, wherein said human coagulation factor is selected from the group consisting of factor V, factor VII, factor IX, factor X, factor XI, factor XII, factor XIII, prothrombin, prethrombin 2, thrombin, fibrin, fibrinogen, tissue factor, von Willebrand factor, prekallikrein, HMW kininogen and combinations thereof.

26. The method as recited in claim 19, wherein said human coagulation factor is selected from the group consisting of factor VIII, factor XIII, factor IX, prothrombin, prethrombin 2, thrombin and combinations thereof.

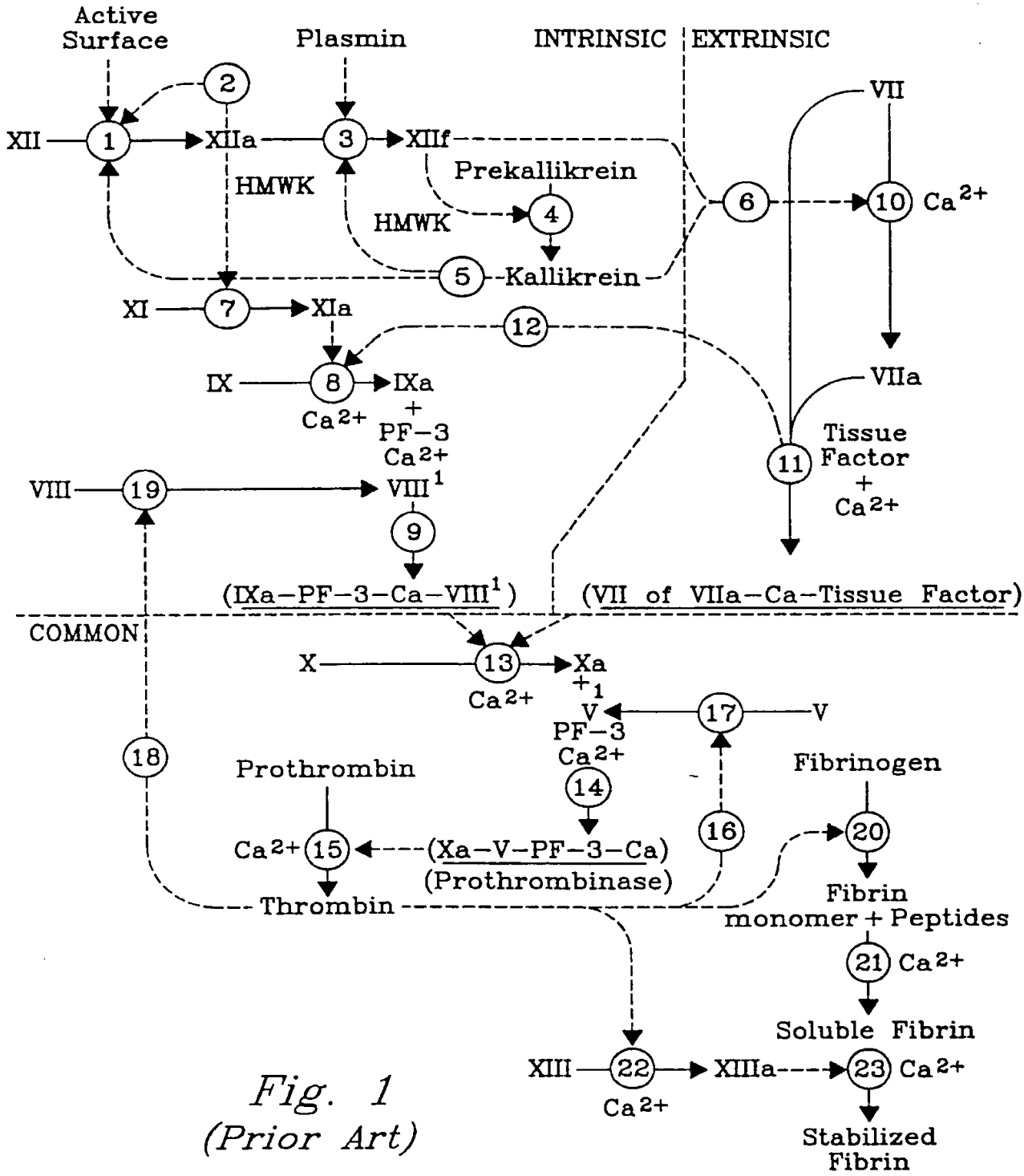

Fig. 1
(Prior Art)

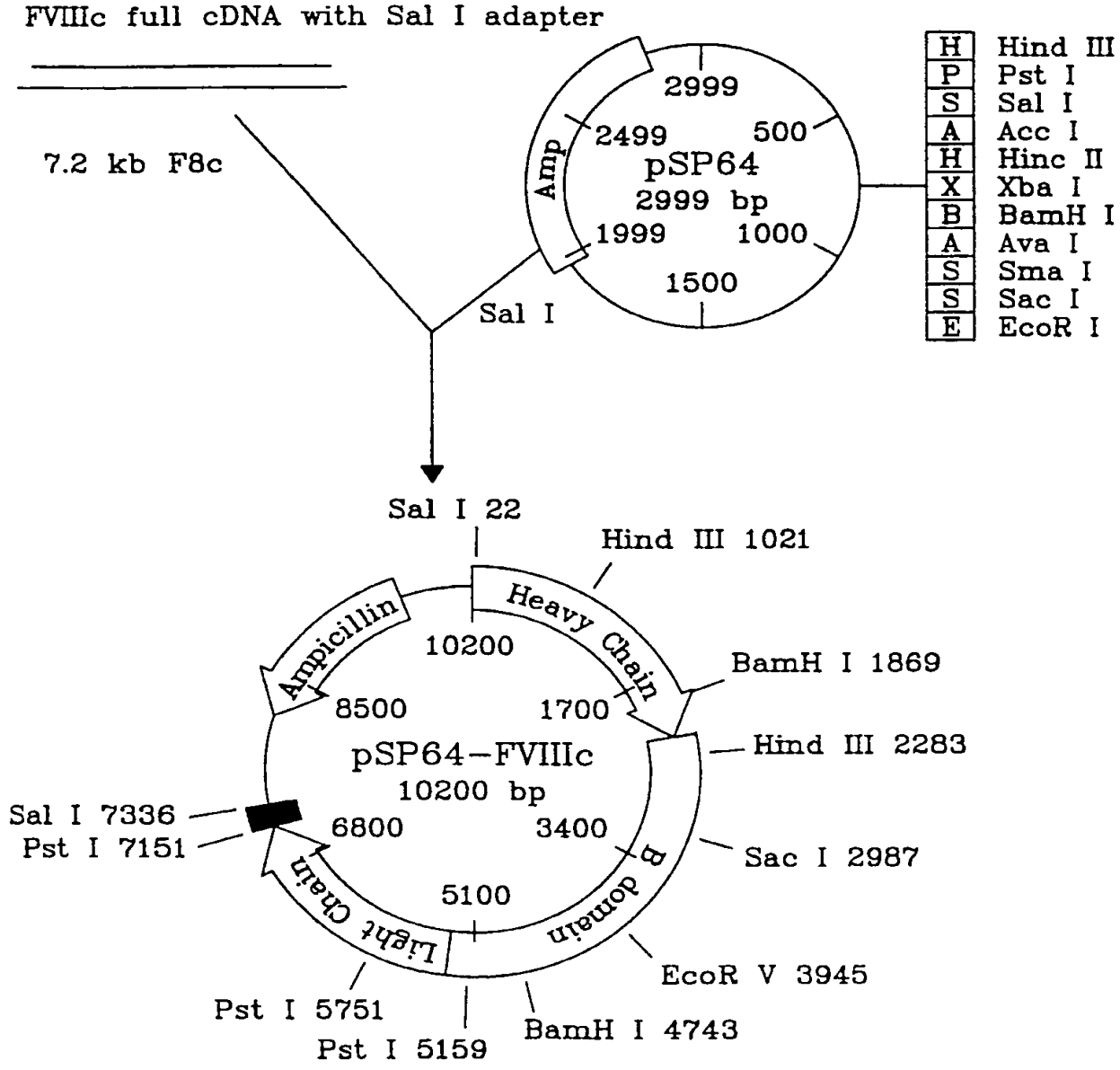

Fig. E1-1

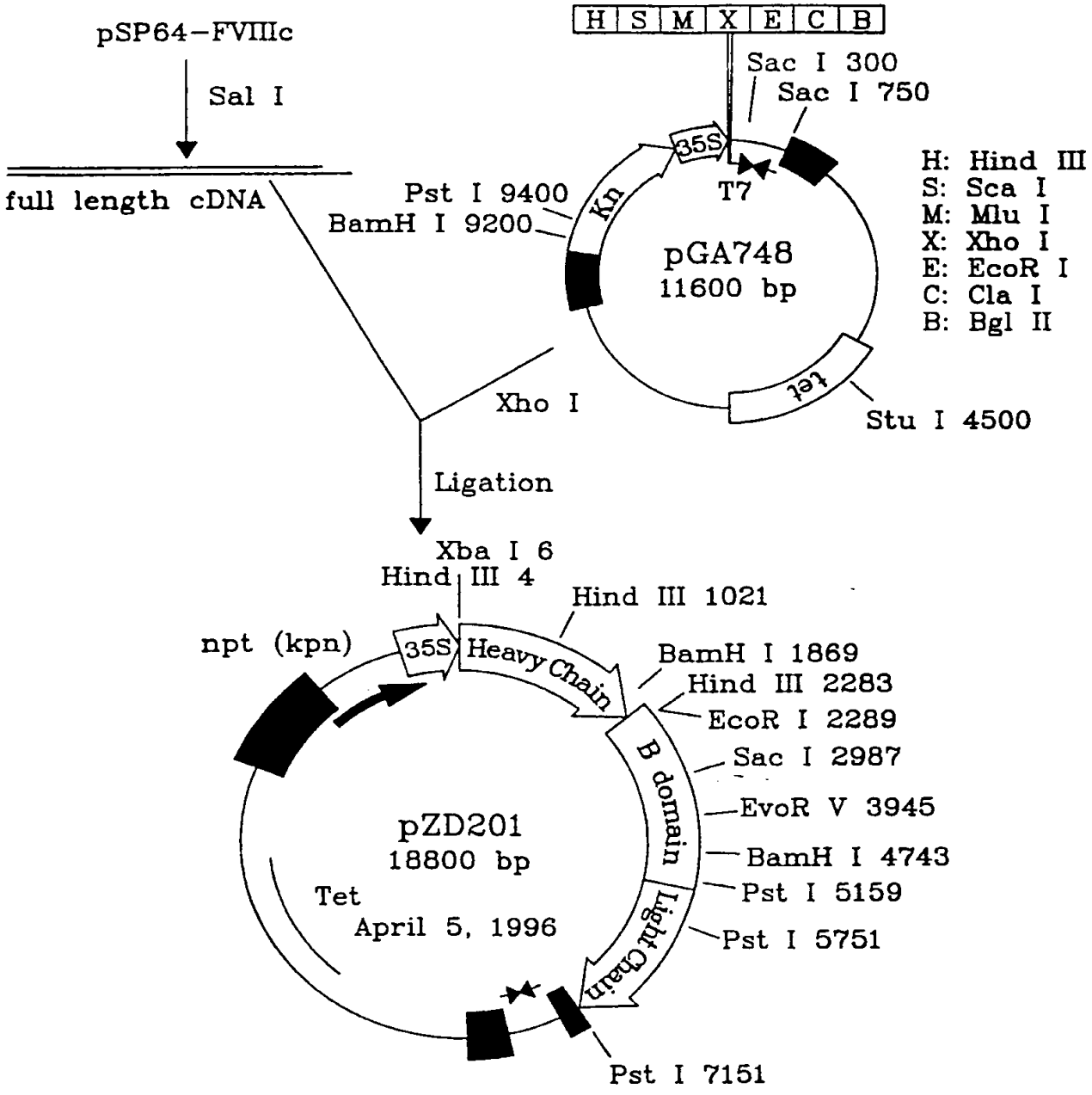

Fig. E1-2

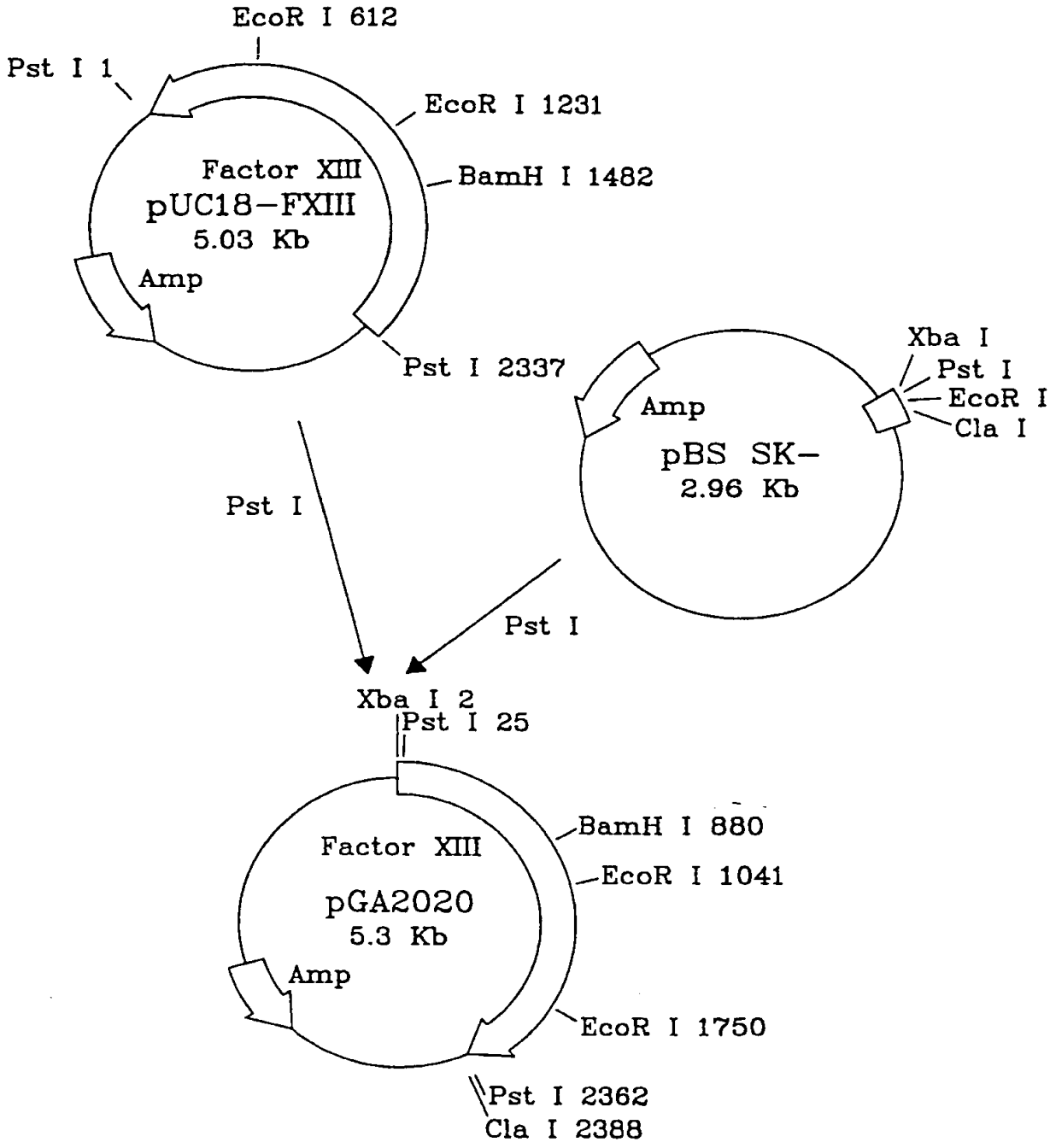

Fig. E1-3

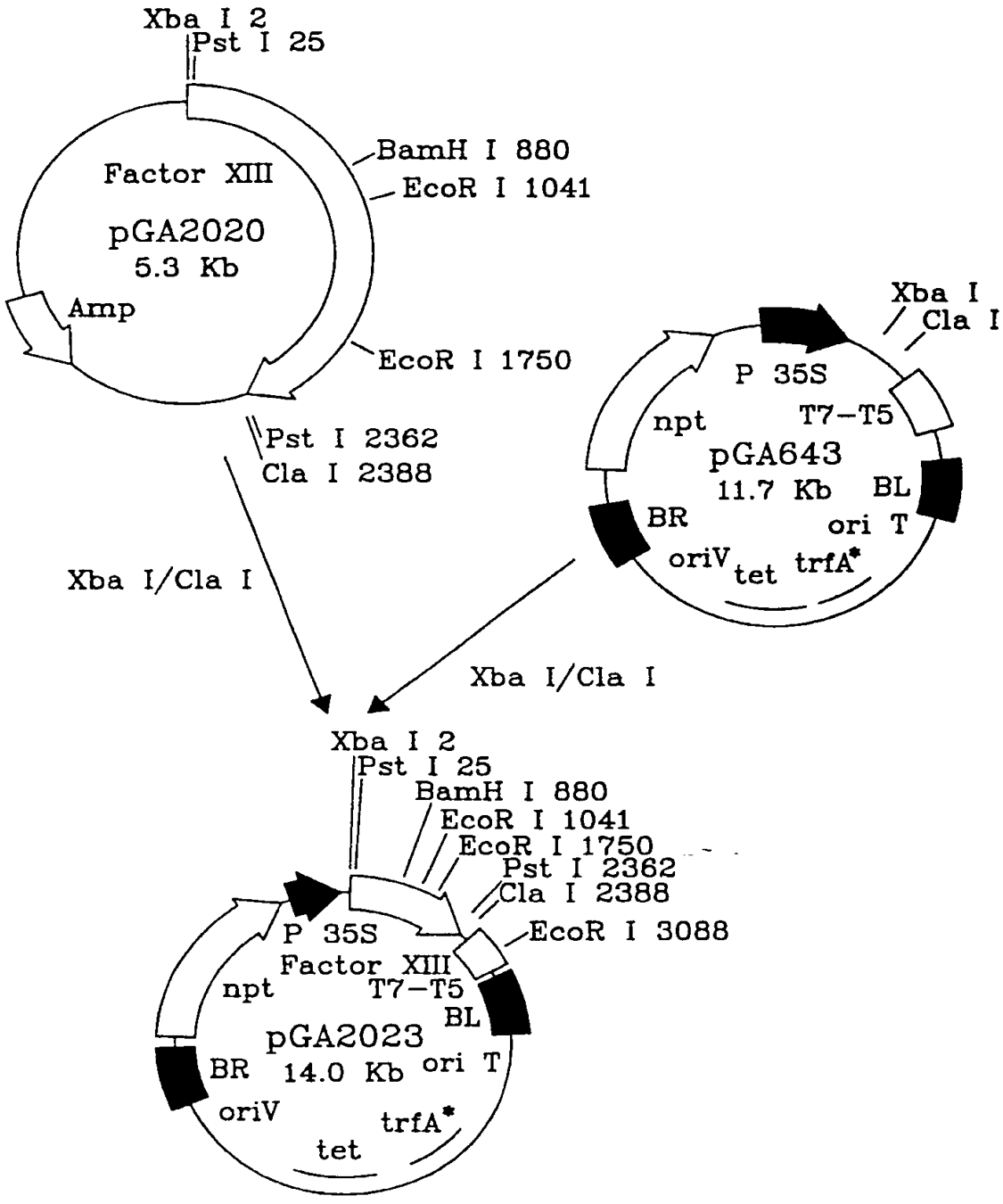

Fig. E1-4

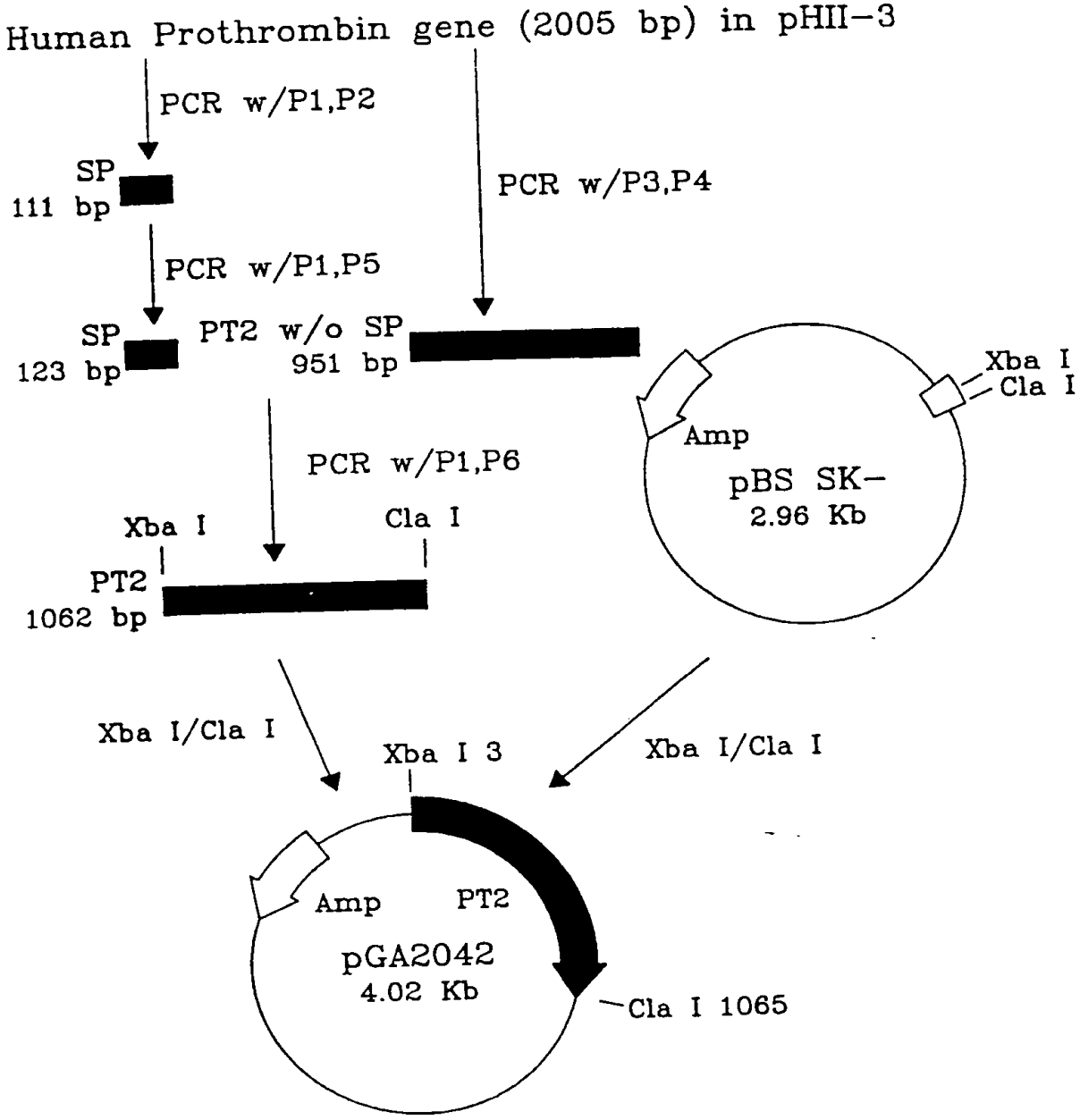

Fig. E1-5

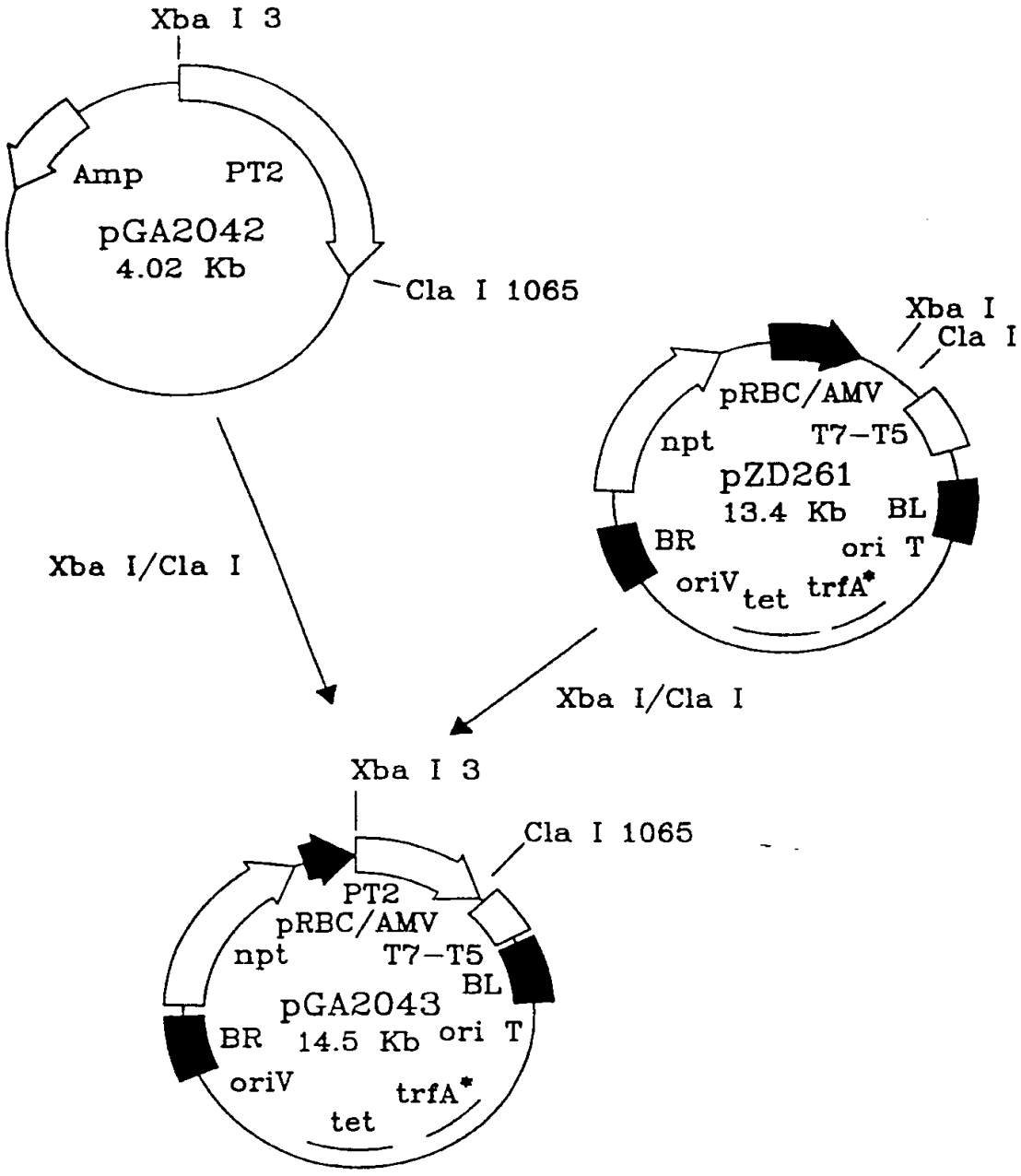

Fig. E1-6

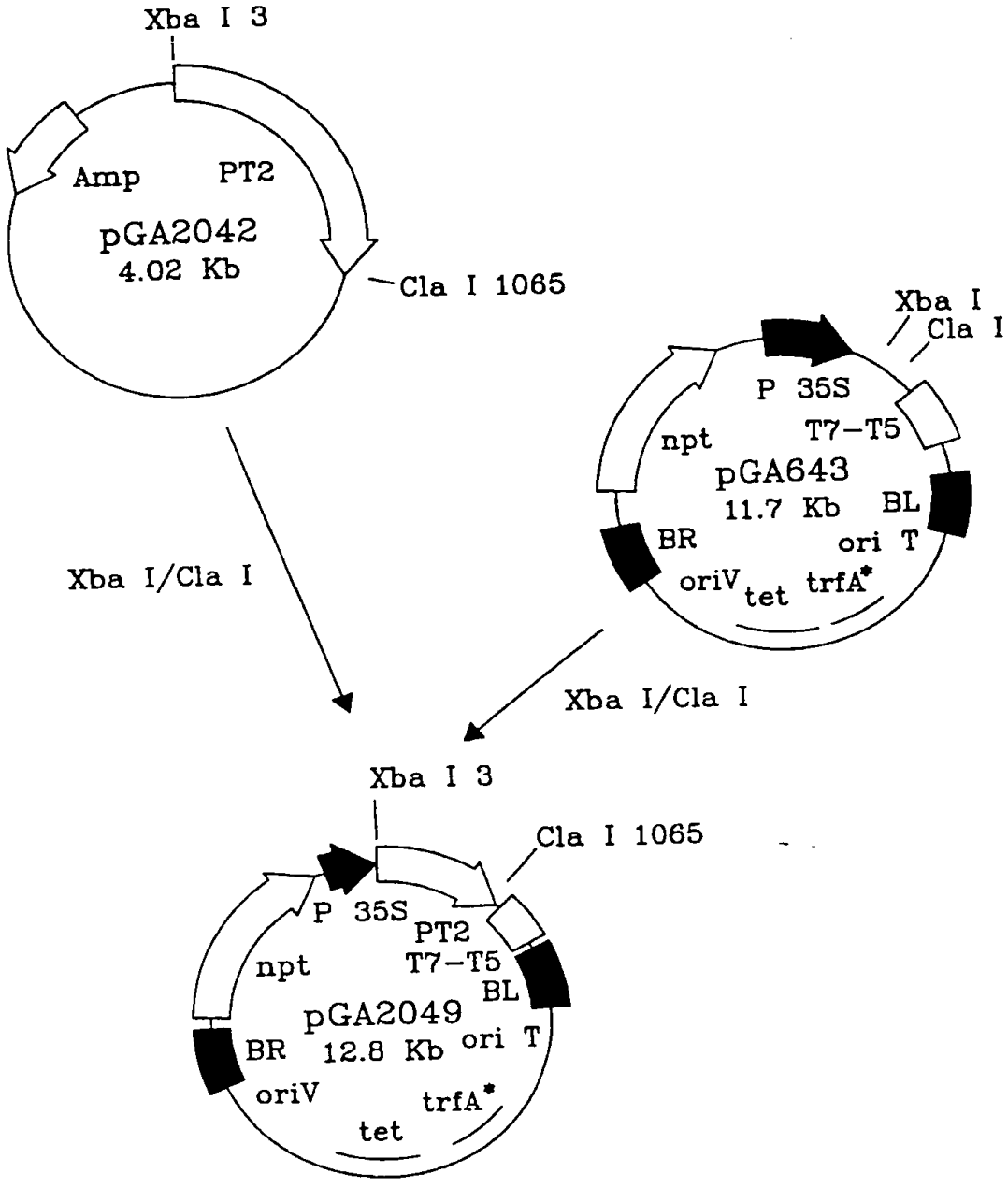

Fig. E1-7

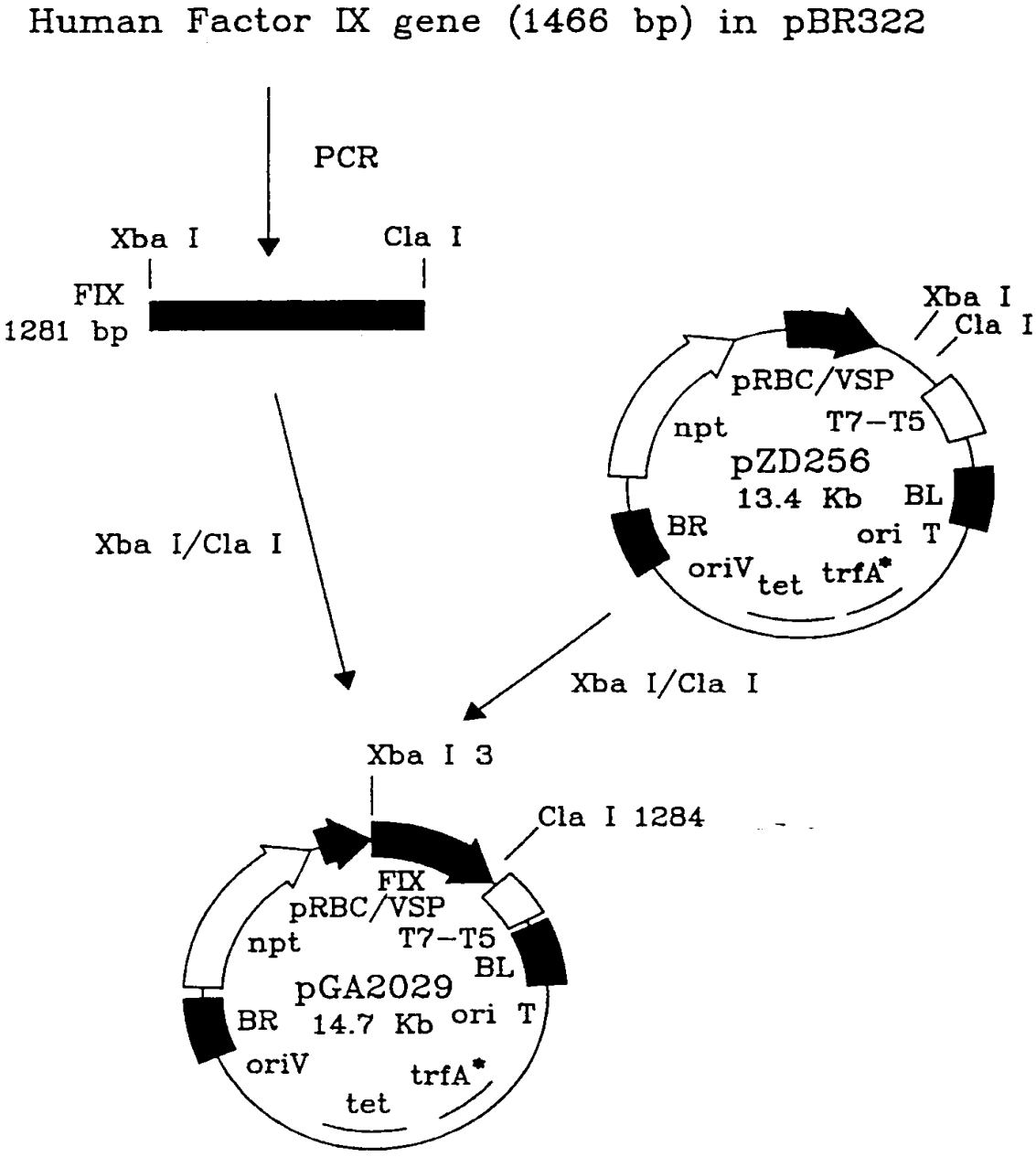

Fig. E1-8

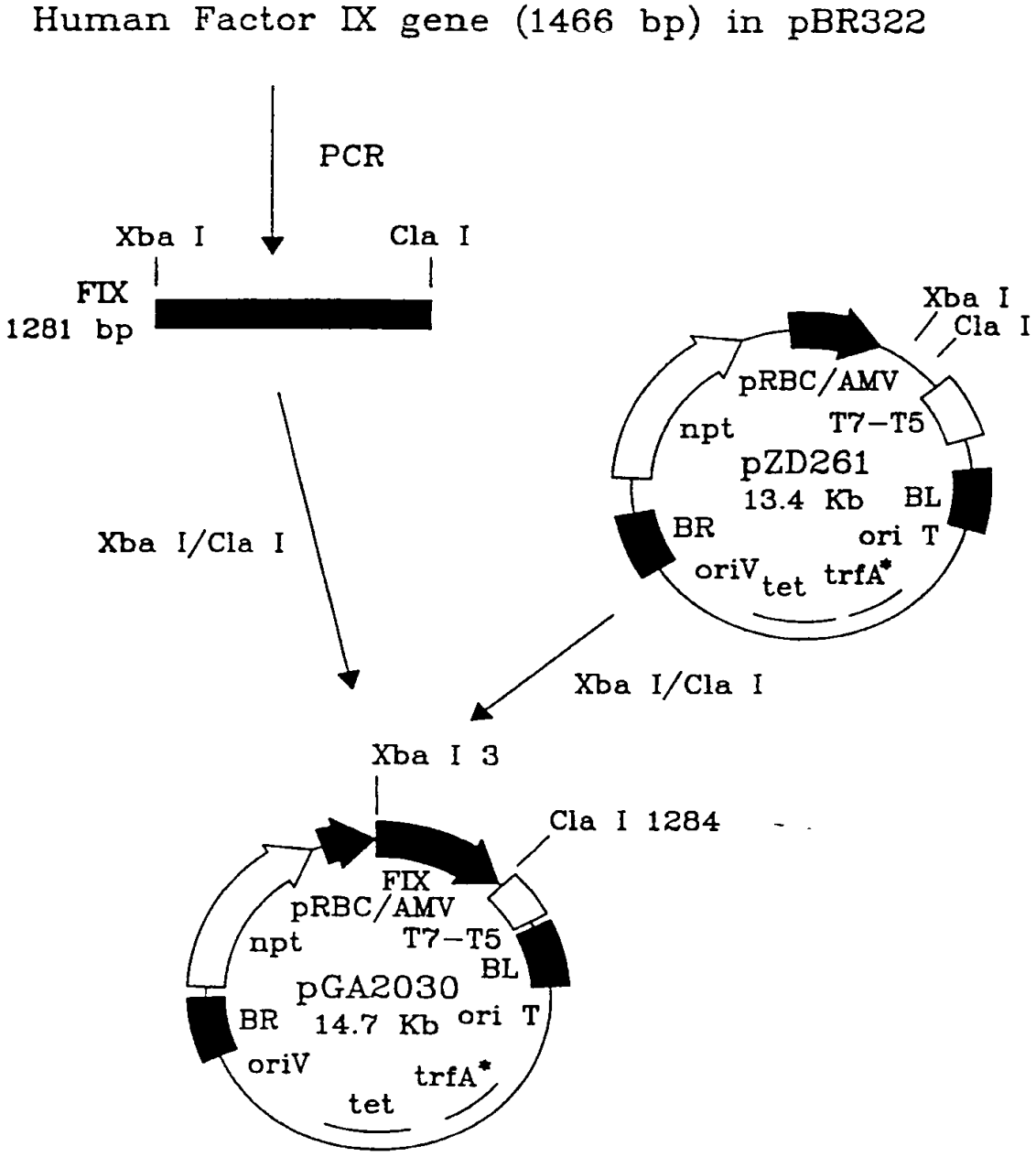

Fig. E1-9

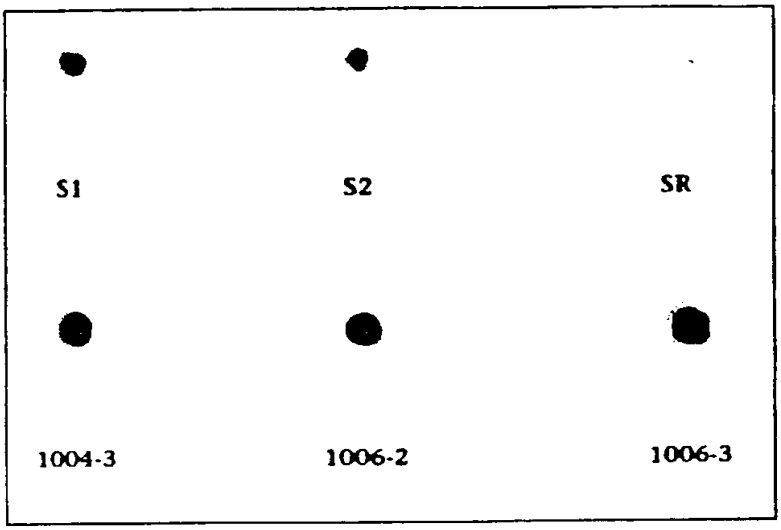

Fig. E3-1

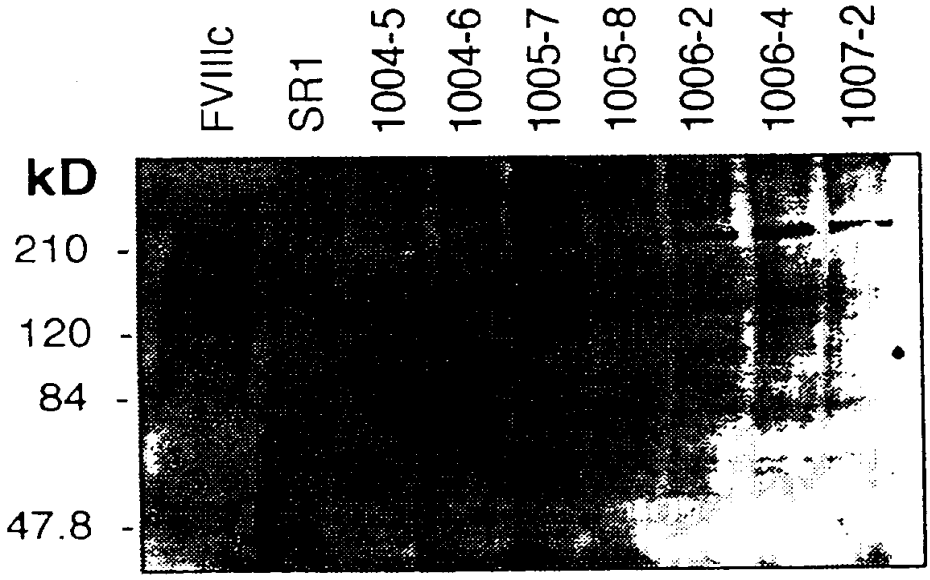

Fig. E3-2

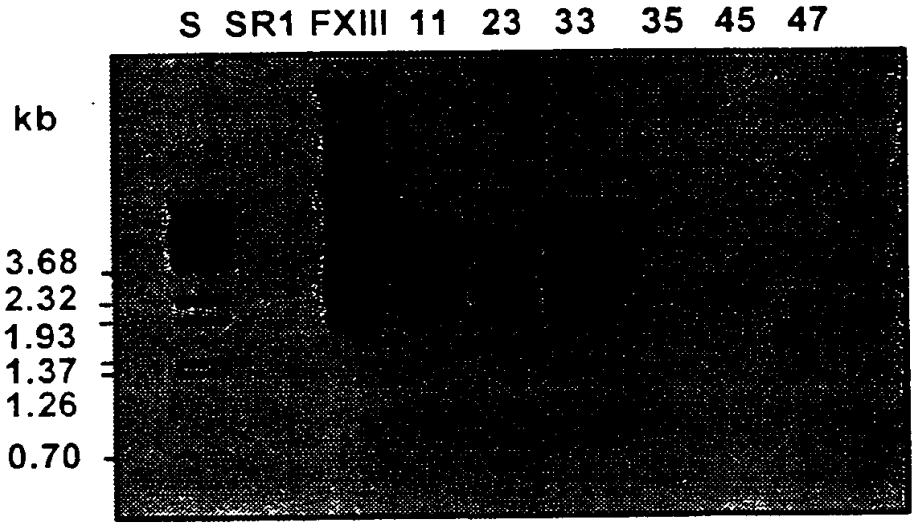

Fig. E4-1

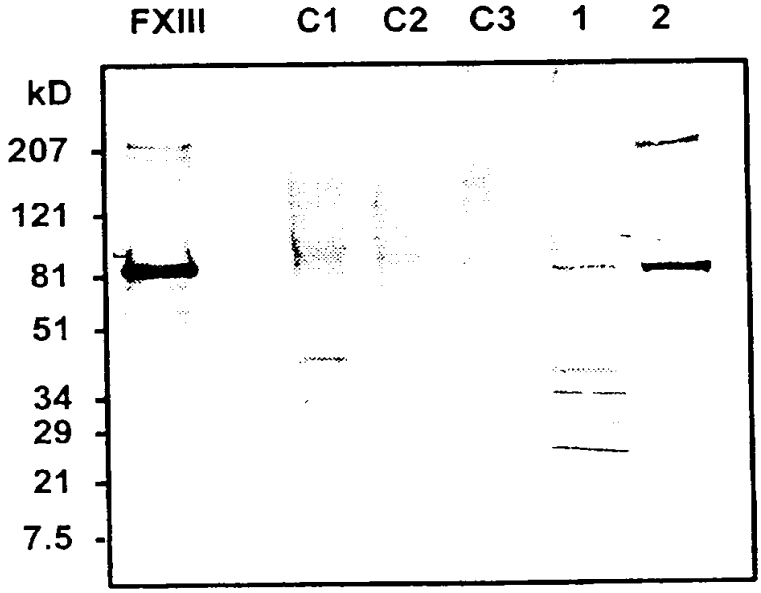

Fig. E4-2

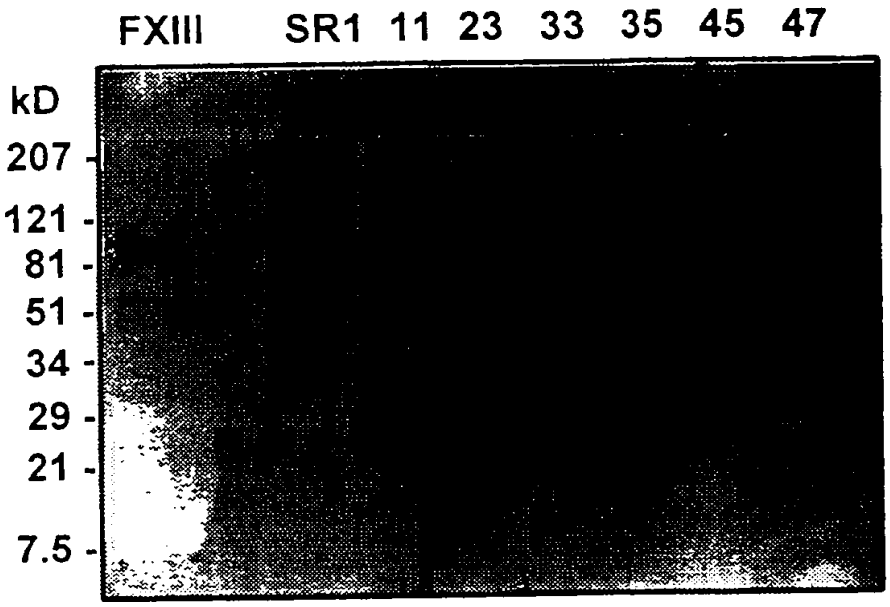

Fig. E4-3

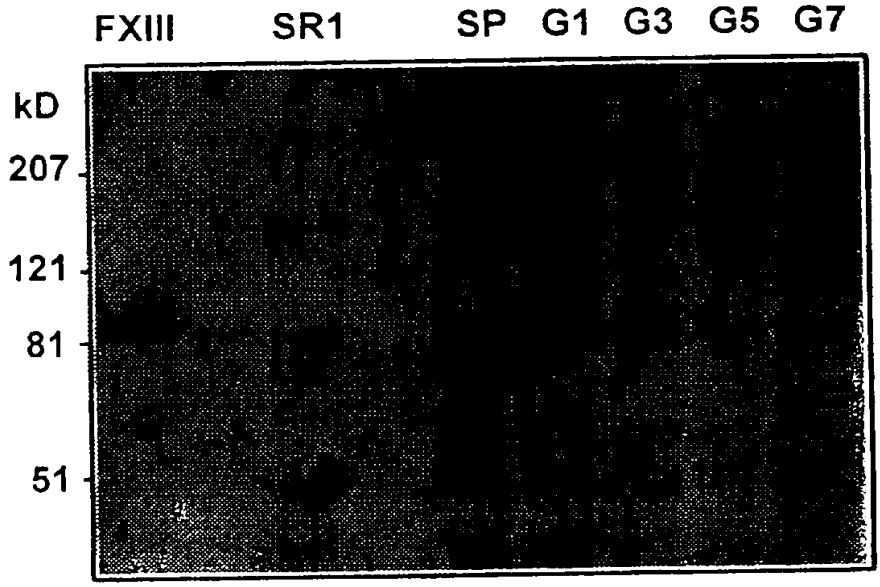

Fig. E4-4

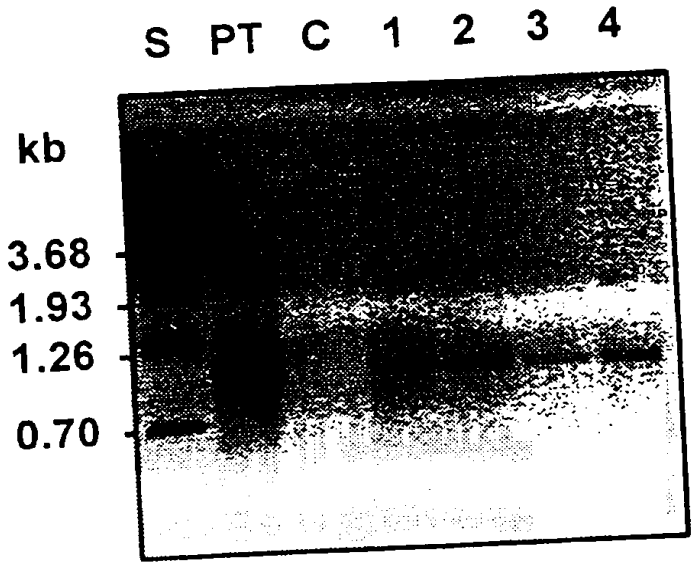

Fig. E5-1

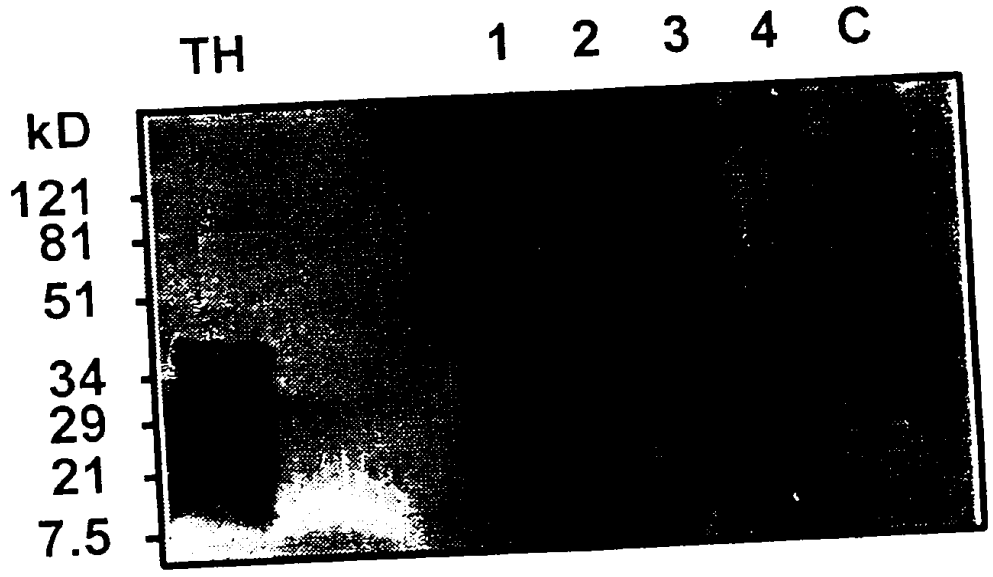

Fig. E5-2

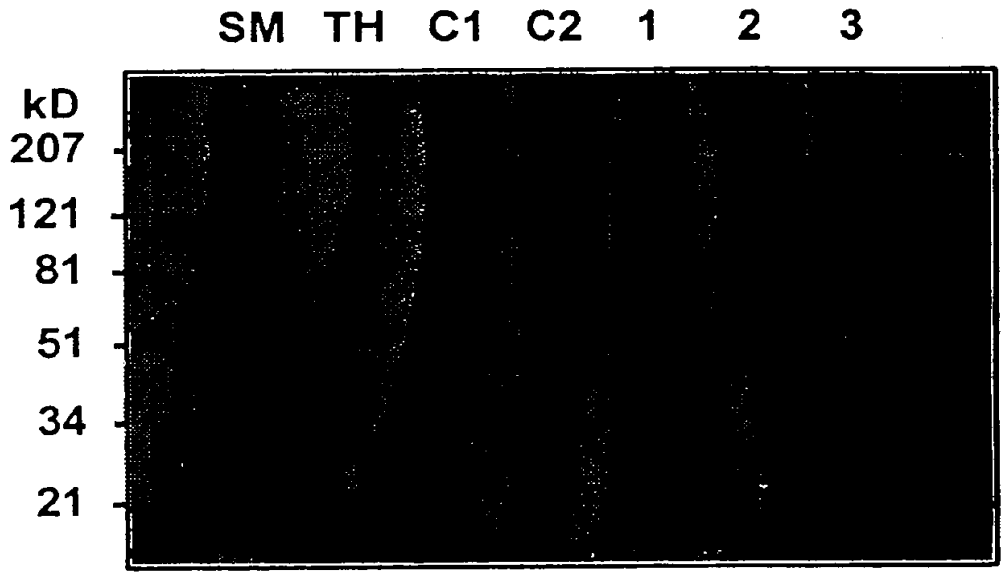

Fig. E5-3

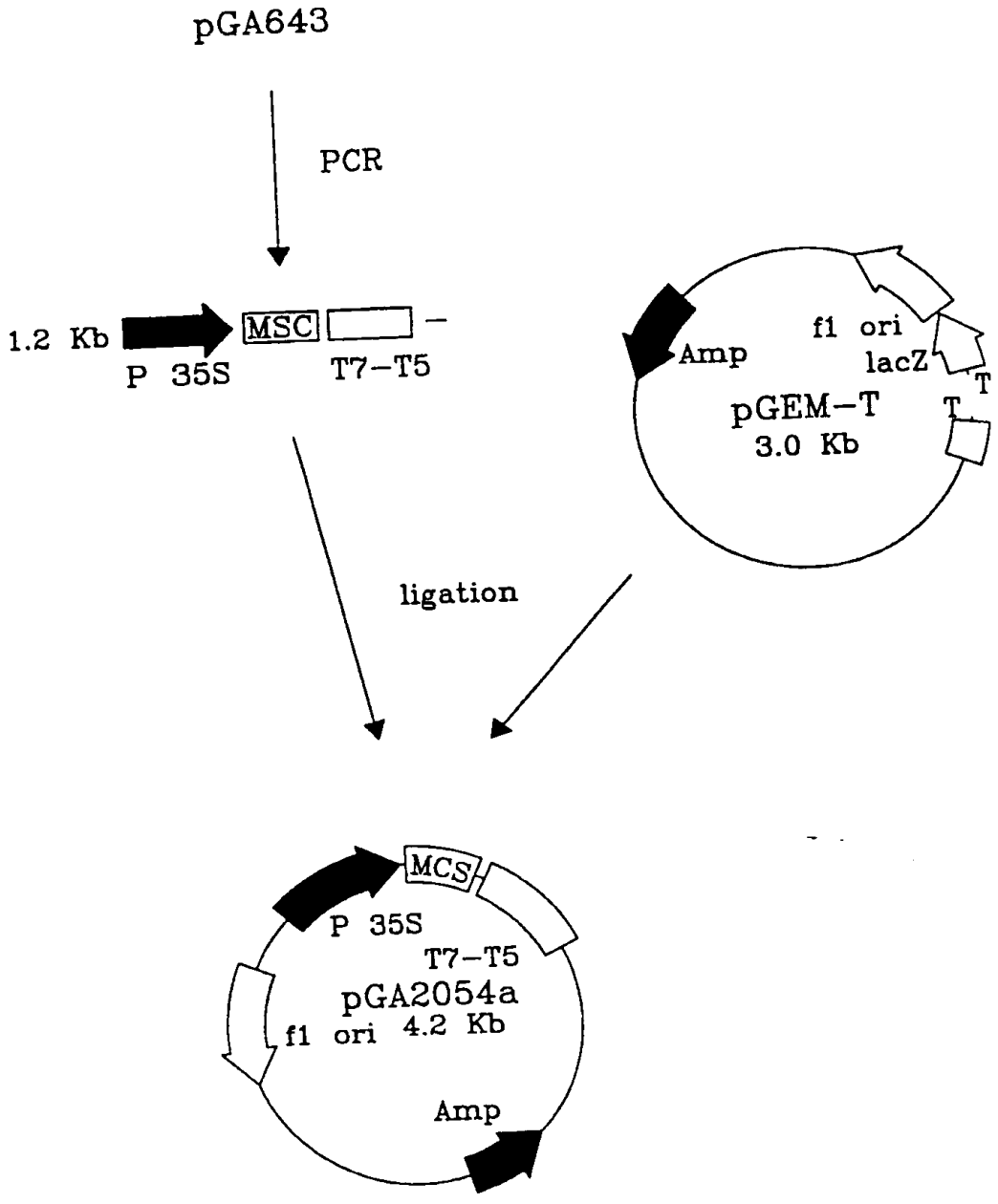

Fig. E5-4

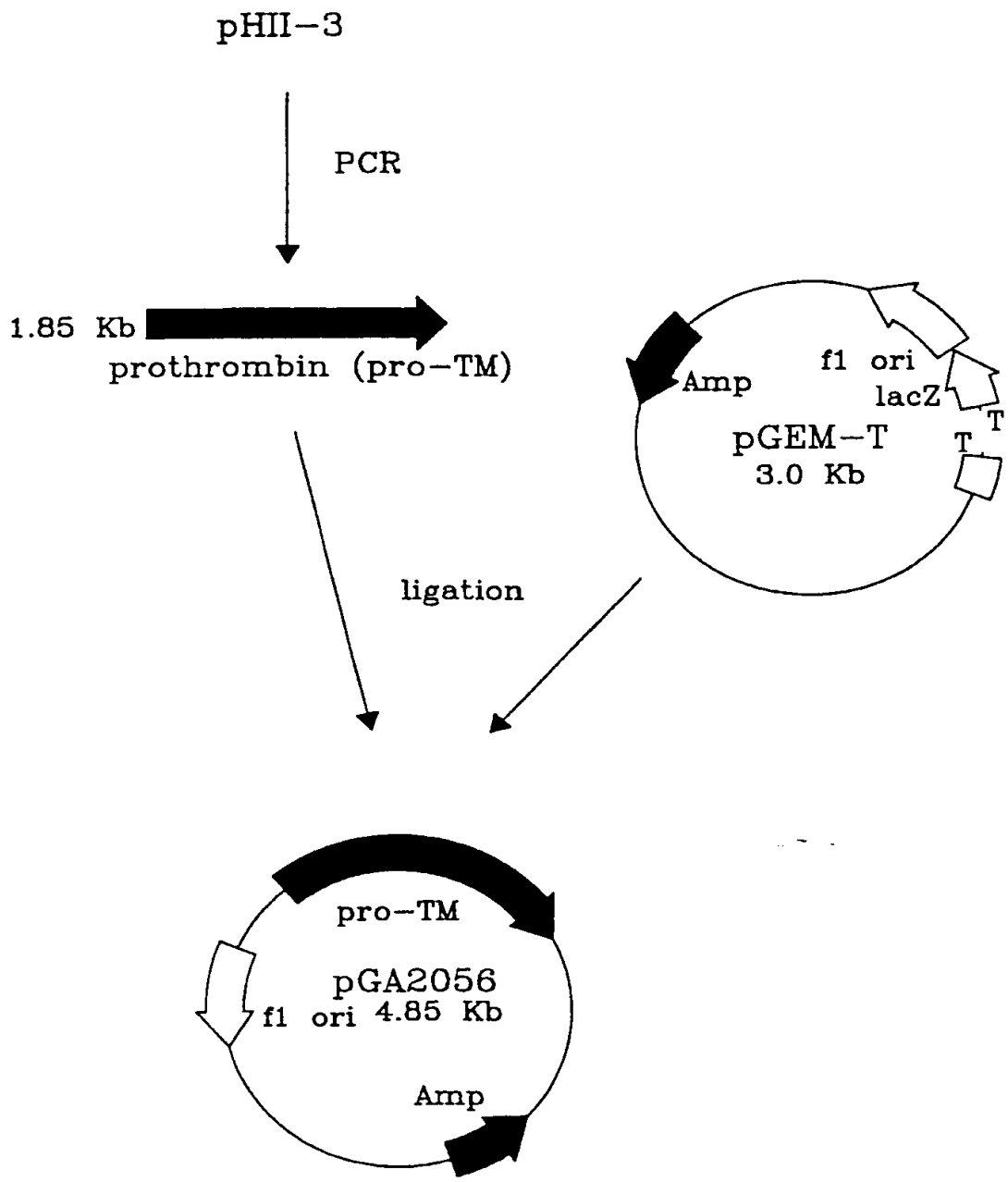

Fig. E5-5

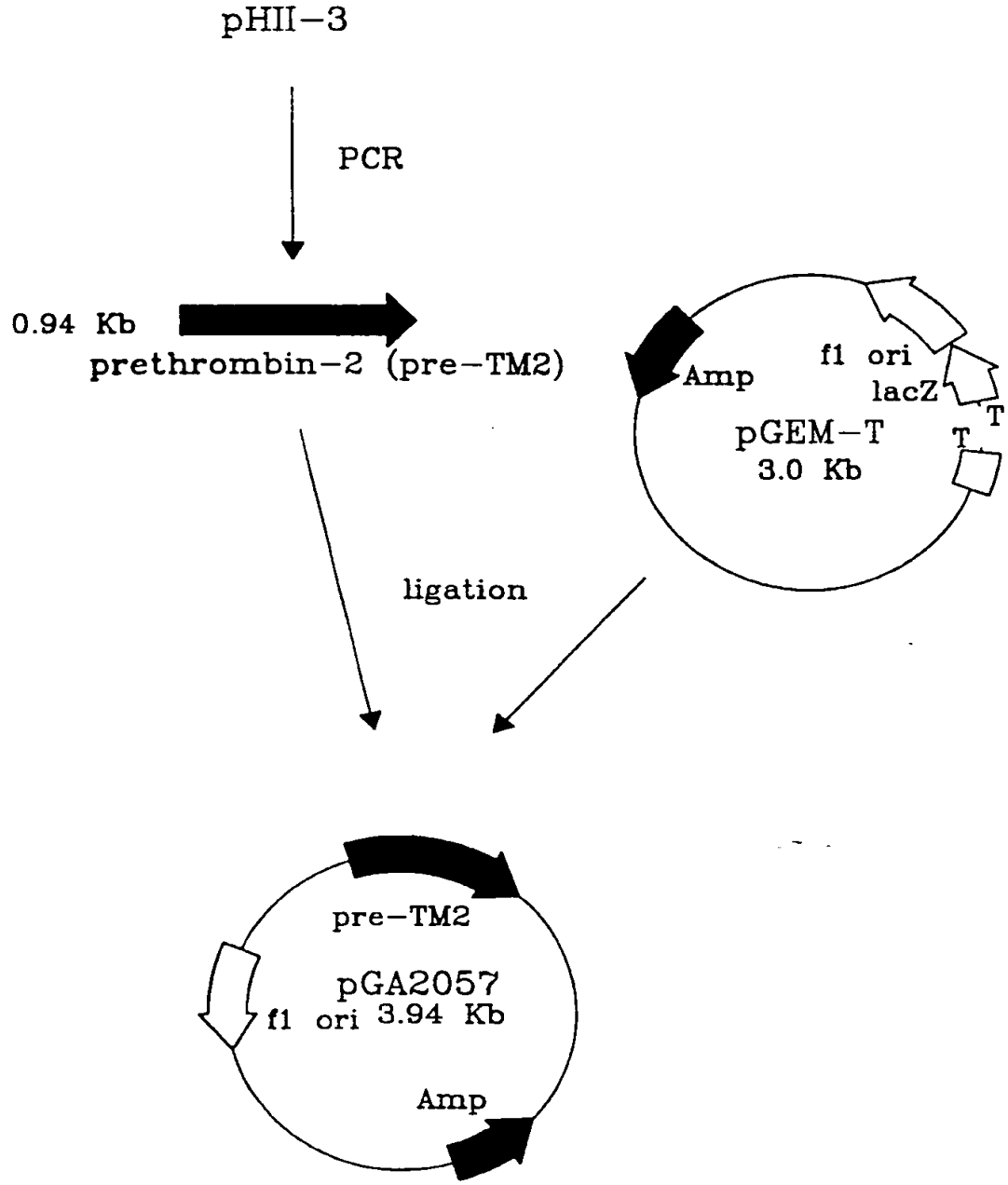

Fig. E5-6

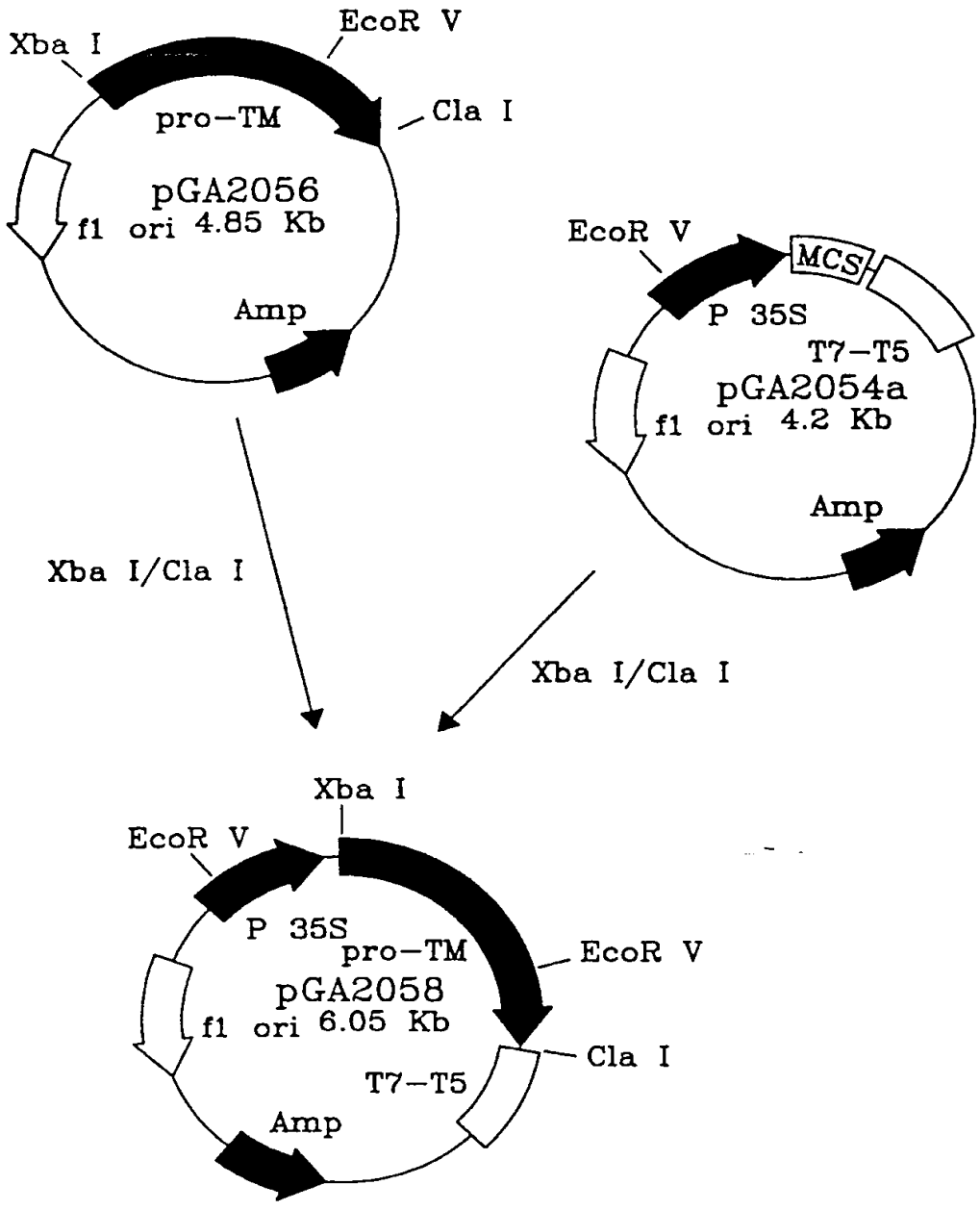

Fig. E5-7

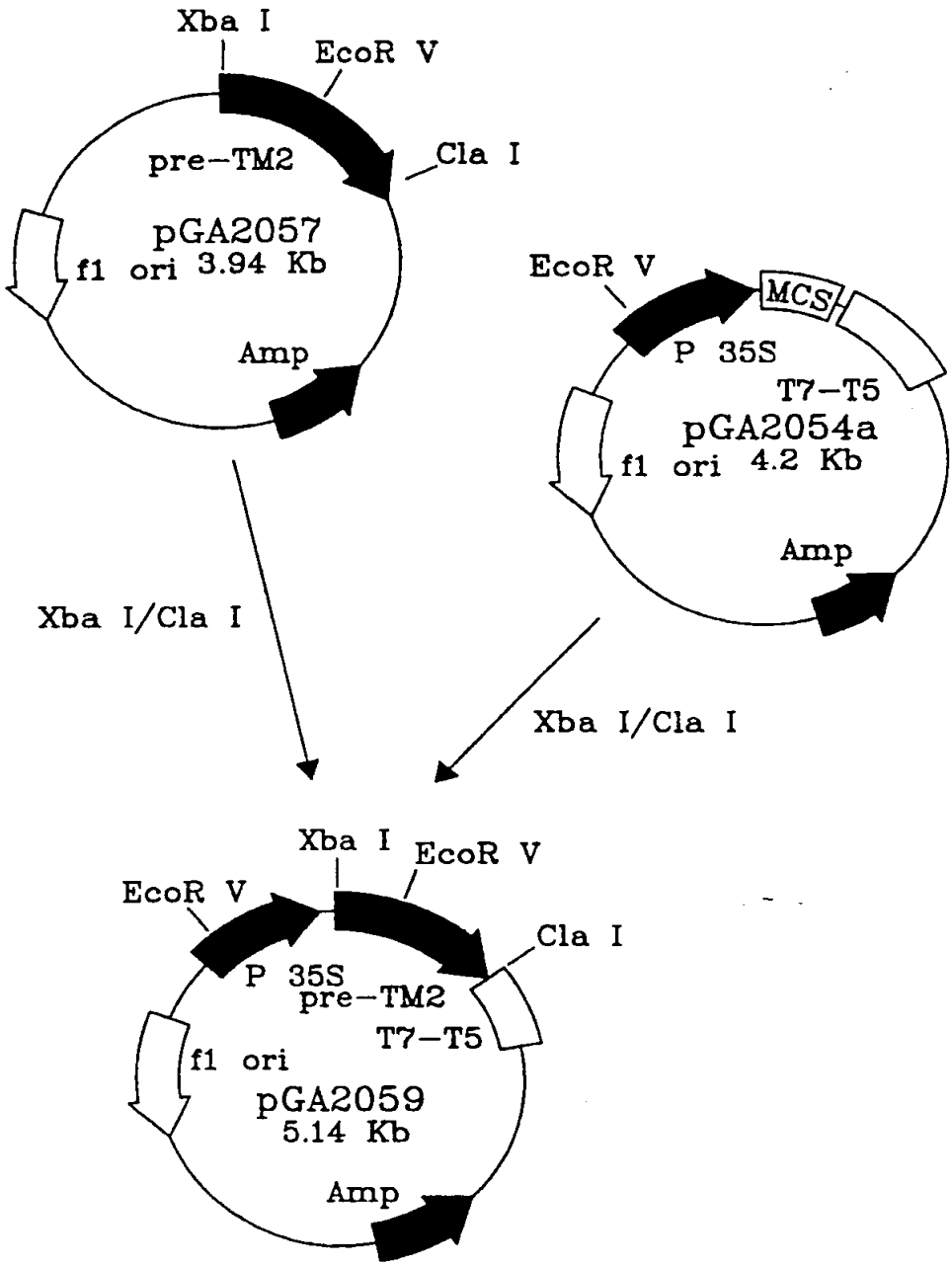

Fig. E5-8

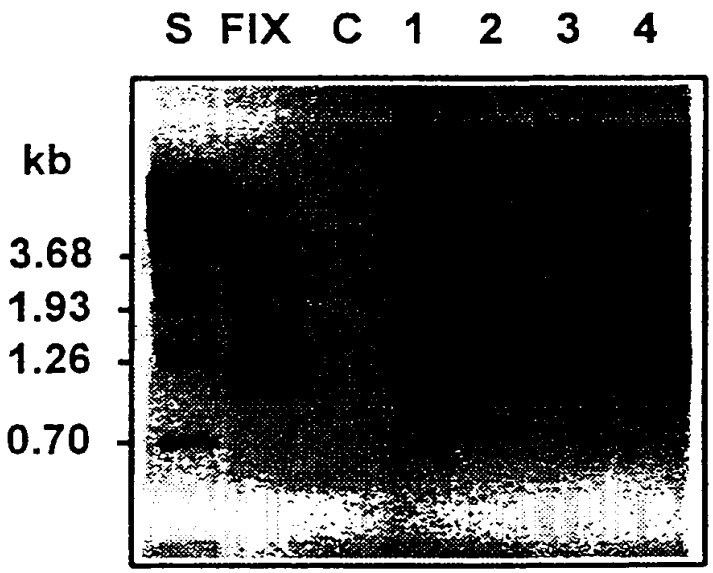

Fig. E6-1

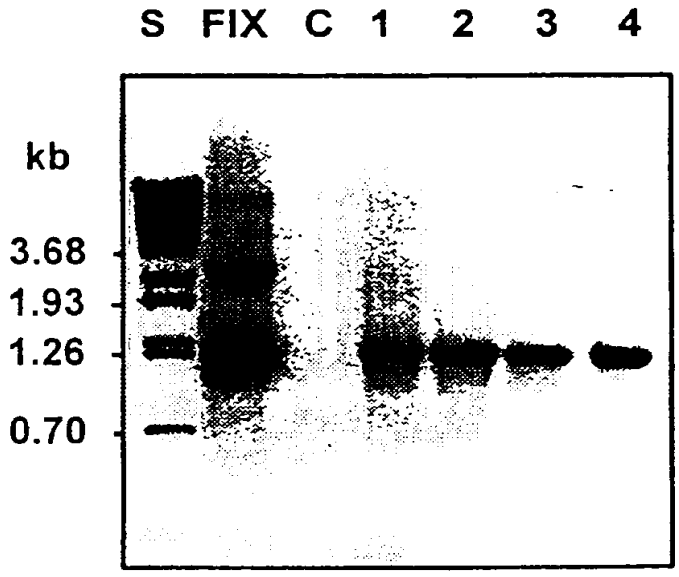

Fig. E6-2

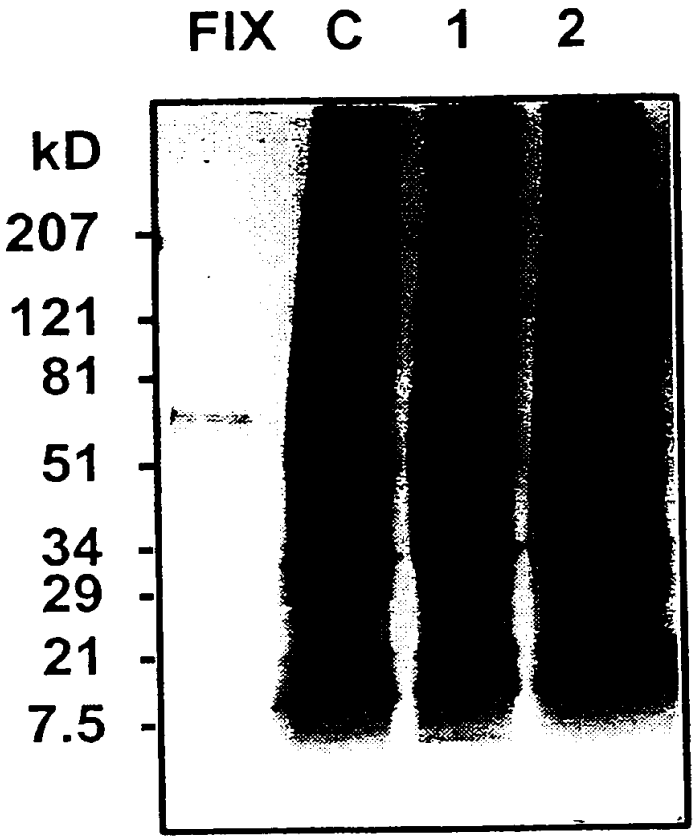

Fig. E6-3

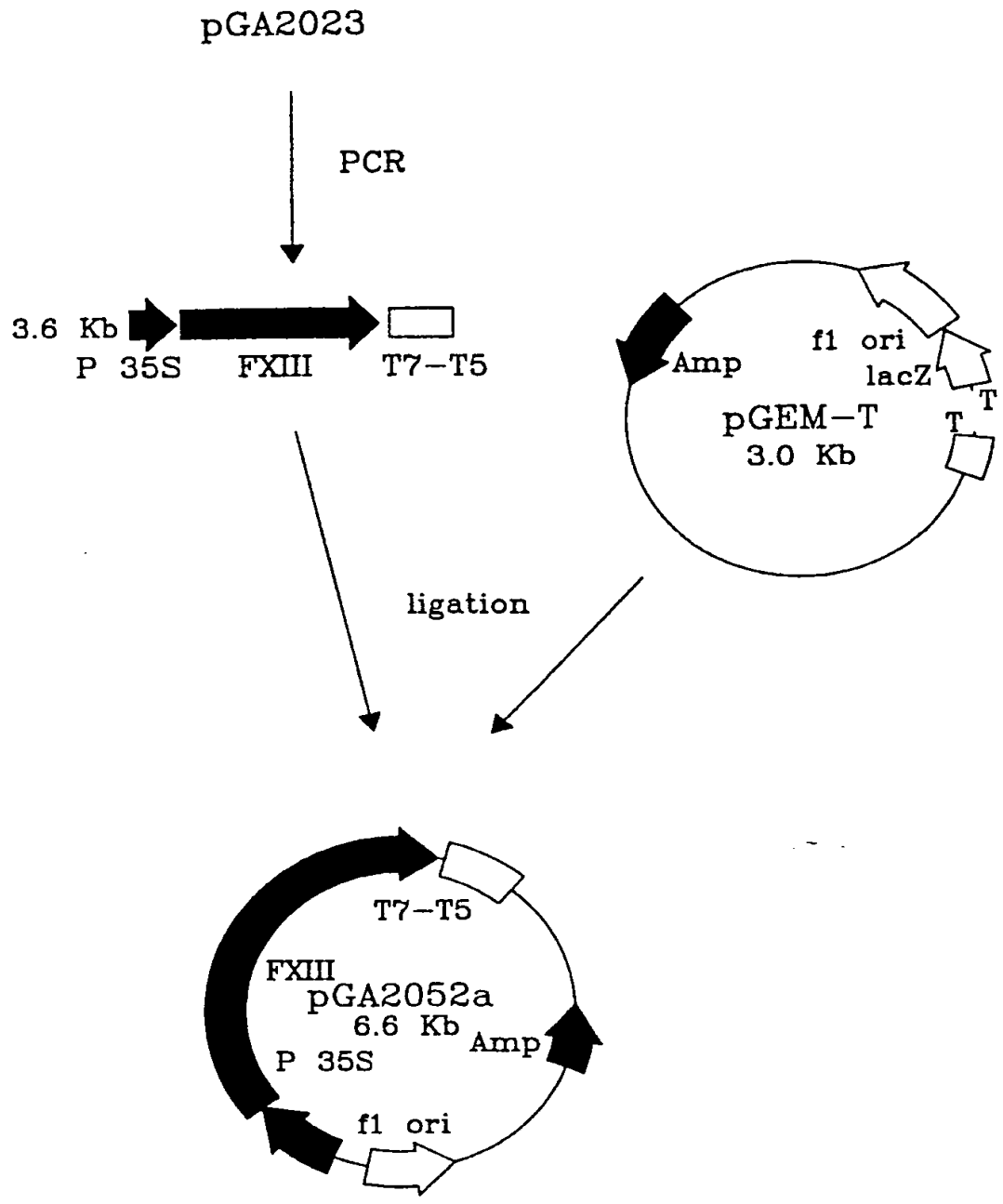

Fig. E8-1

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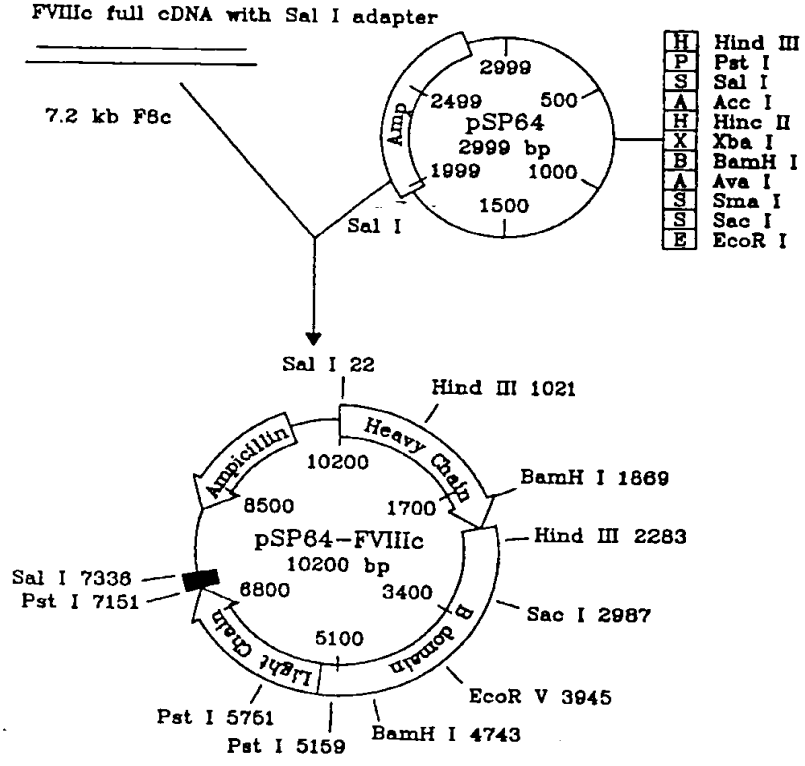
(54) Title: TRANSGENIC PLANT–DERIVED HUMAN BLOOD COAGULATION FACTORS

(57) Abstract

A composition is provided for transgenic plants and transgenic plant derived human coagulation factors *capable of eliciting an activation response in human blood clotting pathways and therefore* useful for the treatment of human beings diagnosed to be deficient in blood clotting factor proteins. Such proteins may be manufactured by methods resulting in viral free production using both whole plants and plant cell cultures. Also provided are expression vectors for the proper transformation of plant tissue for the production of such factors, as well as transformed plant cells and processes for producing human coagulation factors using plant molecular biology techniques.



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/10732

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/82 A01H5/00 A61K38/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 268 772 A (ZYMOGENETICS INC ;UNIV WASHINGTON (US)) 1 June 1988 (1988-06-01) the whole document	1-6
X	WO 93 13208 A (ZYMOGENETICS INC) 8 July 1993 (1993-07-08) the whole document	1-6
X	KANE W H ET AL: "Expression and characterization of recombinant human factor V and a mutant lacking a major portion of the connecting region." BIOCHEMISTRY, (1990 JUL 24) 29 (29) 6762-8., XP002123653 the whole document	1-3,5,6

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2000	24.02.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Kania, T

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Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3,5-8,10-12,14-25 partially

A coagulation factor capable of eliciting an activation response in the human blood clotting pathways, comprising a blood clotting protein derived from a transgenic plant comprising and expressing a DNA sequence coding therefor. Said factor optionally being free of human viral pathogens, said plants being selected from dicotyledonous and monocotyledonous angiosperms. Said factor being factor V. Transgenic plants comprising and expressing said DNA sequence encoding said factor.
A method of eliciting an activation response in human blood clotting pathways using said coagulation factor extracted from said transgenic plants and administering it to a human patient in order to induce an activation response.
Methods for producing from a plant a human like coagulation factor free from human viral pathogens. Said methods being embodied as claimed.

2. Claims: 1-3,5-8,10-12,14-25 partially

idem for factor VII

3. Claims: 1-26 partially

idem for factor IX

4. Claims: 1-3,5-8,10-12,14-25 partially

idem for factor X

5. Claims: 1-3,5-8,10-12,14-25 partially

idem for factor XI

6. Claims: 1-3,5-8,10-12,14-25 partially

idem for factor XII

7. Claims: 1-26 partially

idem for factor XIII

8. Claims: 1-26 partially

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

idem for prothrombin

9. Claims: 1-26 partially

idem for prethrombin 2

10. Claims: 1-26 partially

idem for thrombin

11. Claims: 1-3,5-8,10-12,14-25 partially

idem for fibrin

12. Claims: 1-3,5-8,10-12,14-25 partially

idem for fibrinogen

13. Claims: 1-3,5-8,10-12,14-25 partially

idem for tissue factor

14. Claims: 1-3,5-8,10-12,14-25 partially

idem for van Willebrand factor

15. Claims: 1-3,5-8,10-12,14-25 partially

idem for prekallikrein

16. Claims: 1-3,5-8,10-12,14-25 partially

idem for HMW kininogen

17. Claims: 1,2,4-7,9-11,13-24,26 partially

idem for factor VIII

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/10732

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